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Insights into the epidemiology of enteropathogens of young pigs raised in Cuban piggeries

Pedro Yoelvys de la Fé Rodríguez

Promotor:

Prof. Dr. Bruno Maria Goddeeris

Co-promotoren:

Prof. Dr. Eric Cox en Prof. Dr. Luis O. Maroto Martin

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List of abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AIDA-I	adhesin involved in diffuse adherence
B. coli	*Balantidium coli*
C. parvum	*Cryptosporidium parvum*
C. perfringens	*Clostridium perfringens*
CPE	*C. perfringens* enterotoxin
DAS-ELISA	double-antibody-sandwich enzyme-linked immunosorbent assay
E. coli	*Escherichia coli*
EAST-I	enteroaggregative *E. coli* heat stable enterotoxin
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
EPEC	enteropathogenic *E. coli*
ETEC	enterotoxigenic *E. coli*
FAT	fluorescent antibody technique
F18R	F18 receptor
F4R	F4 receptor
I. suis	*Isospora suis*
IC	immunochromatography
IF	immunofluorescence
IFAT	indirect fluorescence antibody technique
IHC	immunohistochemistry
Int	intimin
ISH	*In-situ* hybridization
kDa	kilo Dalton
LT	heat-labile enterotoxin
MAb	monoclonal antibody
OD	optical density
PAA	porcine attaching and effacing-associated factor
PAGE	polyacrylamide gel electrophoresis
pAPN	porcine aminopeptidase N
PCR	polymerase chain reaction

PCR-RFLP	PCR-restriction fragment length polymorphism
PEDV	porcine epidemic diarrhea virus
PRCV	porcine respiratory coronavirus
PRRSV	porcine respiratory and reproductive syndrome virus
RT-PCR	reverse transcriptase PCR
ST	heat-stable enterotoxin
TGE	transmissible gastroenteritis
TGEV	transmissible gastroenteritis virus
VN	virus neutralization
VTEC	verocytotoxigenic *E. coli*

Introduction

Porcine pre- and post-weaning diarrhea negatively impact the economic feasibility of the swine industry due to mortality, costs of medication, and growth retardation (Francis, 1999; Katsuda *et al.,* 2006; Ushida *et al*., 2009). Housing and management conditions (e.g. hygiene, comfort temperature, intake of maternal antibodies, feeding), vaccination against enteric pathogens, and surveillance of antibiotic resistance are crucial aspects to consider during prevention of porcine diarrhea (Fairbrother *et al.,* 2005; Fairbrother, 2006; Straw *et al.,* 2006).

Diarrhea of young pigs is frequently caused or complicated by rotavirus, transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), enterotoxigenic *Escherichia coli* (ETEC), toxigenic *Clostridium perfringens*, and *Coccidia* (Wieler *et al.,* 2001; Straw *et al.,* 2006; Katsuda *et al.,* 2006). Therefore, the differential identification of infectious agents is necessary to evaluate diarrhea epidemiology in a swine herd (Collins *et al.,* 1989; Niestrath *et al*., 2002; Nuñez *et al.,* 2003). However, reports of surveys aimed at studying the mixed condition of piglet's infectious diarrhea have been scarce worldwide (Wieler *et al.,* 2001; Adesiyun *et al.,* 2001; Yaeger *et al.,* 2002; Katsuda *et al.,* 2006); many studies on porcine diarrhea have covered only single pathogens (Quilez *et al.*, 1996; Osek, 1999; Barreiros *et al.*, 2003), and although in a TGEV/PEDV prevalence survey, also pathogenic bacteria were identified, results were not shown (Chae *et al.,* 2000). When performing experiments related with the porcine digestive tract, the differential identification of enteropathogens should be carried out as enteropathogens can significantly influence the results (Jensen *et al.,* 2006; Niestrath *et al.,* 2010). Furthermore there is urgent need to better identify synergisms or antagonisms among these pathogens (Baba and Gaafar, 1985; Choi *et al.,* 2003).

ETEC are the most common cause of diarrhea in suckling and recently weaned pigs (Katsuda *et al.,* 2006). Cheng *et al.* (2006), Zhang *et al.* (2007), Madoroba *et al.* (2009), and Vidotto *et al.* (2009) found that F4 or F18 fimbriae were the major fimbrial antigens expressed by pathogenic *E. coli* associated with swine diarrhea. These fimbriae mediate the adhesion of ETEC to receptors present on the surface of enterocytes, favoring gut colonization. The subsequent production of enterotoxins by these bacteria leads to the secretion of electrolytes and water across the mucosa, resulting in a watery diarrhea. Additionally, F18 favors the adhesion of verocytotoxigenic *E. coli* (VTEC) which cause edema disease through a systemic vascular damage provoked by the verocytotoxin STx2e (Fairbrother, 2006; Fairbrother and Gyles, 2006; Oanh *et al.,* 2010).

Swine production is very important in Cuba as pork is the most consumed meat. In 2005 1,980,000 pigs were slaughtered and five years later already 3,266,600 according to the National Office for Statistics (ONE, 2011).

In Cuban piggeries diarrhea is common and strongly reduces the survival rate of young pigs leading to considerable economic losses: diarrheic diseases are responsible for 31% and 37% of the total mortality during the pre- and post-weaning periods, respectively (Cabrera and García, 2009). For instance, in the whole country 506,400 suckling piglets died in 2009 (12.2% mortality; ONE, 2011), and the 31% mortality provoked by diarrhea in this age group represent 156,984 deaths (Cabrera and García, 2009).

It is contradictory that in Cuba specific and updated epidemiological information related to swine enteropathogens is scarce. The prevention and control of diarrhea in Cuban piggeries are not always well conducted due to lack of infrastructure in the Provincial Veterinary Diagnostic Laboratories to properly perform the identification of enteropathogens (Cabrera *et al*., 2010).

Pedroso and Talavera (1983) showed in 1983 the presence of $F4^+$ and $F5^+$ *E. coli* in feces of piglets in the Havana province, whereas Blanco *et al.* (2006) could not isolate $F4^+$, $F5^+$ or $F41^+$ *E. coli* from diarrheic piglets in 2002 in the Villa Clara province. Most of their *E. coli* isolates (61%) were $F18^+$. The prevalence of *Cryptosporidium parvum* and *Isospora suis* was studied more than 20 years ago in the Havana province in diarrheic piglets and was 2.1% and 44.7%, respectively (Cabrera and García, 1985; Koudela *et al.,* 1989). The first outbreak of epidemic transmissible gastroenteritis (TGE) in Cuba was reported in Havana in 2003 (Barrera *et al.,* 2005).

The present thesis contributes to the epidemiological characterization of enteropathogens associated with porcine pre- and post-weaning diarrhea in Cuba, with special emphasis on pathogenic *E. coli*. All epidemiological data obtained and discussed herein can be used for the implementation of accurate preventive and therapeutic strategies to control porcine enteropathogens in Cuba.

Chapter 1

General introduction

Cuban context

In 1493, during his second trip to America, Christopher Columbus carried eight Iberian-trunk pigs to Cuba on request of the queen Isabella I of Castile and León (Laguna, 1998). Nevertheless, for more than four centuries during the colonial and neo-colonial periods, the production of cane sugar, alcohol, tobacco, and leather were primordial in Cuba and pork was not an important production. By 1959, 68% of swines were bred extensively. Approximately, 16% of the herds were intensively managed by breeders and the remainder was a family-type production. From a genetic point of view, 22% of swine were pure Criollo, and 69% were Criollo's crossbreds. Only 9% of swine herds had specialized breeds, mainly Hampshire and Duroc. These genetic and management statuses did not allow an efficient swine production leading to a high import dependency from the North American market before 1959 (Rico, 2005).

From the early 60's, as part of the changes that occurred in Cuba due to the 1959 Revolution, swine production benefited from the following strategies:

i- Creation of the swine production department in the Ministry of Agriculture and elaboration of a national policy for pig health and management.
ii- Foreign consultancy and staff training.
iii- Building of farms for intensive swine production and introduction of pure swine breeds into breeding programs monitored by swine genetic centers located all over the country, which ensured specialized swine production.

From 1971 to 1989, artificial insemination started to be applied and swine production was re-organized under a pyramidal structure including genetic centers, multiplication piggeries (which obtained replacement stock animals) and commercial piggeries. Also, swine sector benefited from investments in facilities and from importations (mainly food and medicines) at preferential prices agreed with the Council of Mutual Economic Assistance (COMECON). During this period, about 84% of swine production occurred by the specialized state sector and the remaining 16% by private producers and agricultural cooperatives (Rico, 2005).

From the 90's onwards, Cuban economy was negatively impacted by the dissolution of COMECON due to the collapse of the communism in Eastern Europe and the Soviet Union, which were at that moment the main trade partners and sources for technology transfer. Then, as happened with most institutions, the services of the Cuban Veterinary Medicine Institute as well as swine production reduced

their activity. Specifically, the diagnostic effectiveness decreased and ongoing research on animal health slackened, resulting in insufficient diagnosis of viral and bacterial agents. As a result, information regarding swine enteric pathogens have been limited in Cuba:

- Pedroso and Talavera (1983) detected $F4^+$ and $F5^+$ *E. coli* by immunofluorescence in feces of piglets in the Havana province.
- The prevalence of *C. parvum* in diarrheic piglets from Havana was 2.5% in 1985 (Cabrera and Garcia, 1985).
- 44.7% of diarrheic piglets were infected with *I. suis* in a piggery from Havana in 1989 (Koudela *et al*., 1989).
- Fuentes *et al*. (2001) reported a 2.8% prevalence of $F4^+$ *E. coli* in 212 diarrheic piglets sampled in 1998 in the Camagüey province.
- The first outbreak of TGEV was reported in 2003 in Havana and the disease was later reproduced experimentally (Barrera *et al*., 2005).
- Blanco *et al*. (2006) genotyped *E. coli* isolates from diarrheic piglets in 2002 and found that most of them (61%) carried the F18 encoding gene and that F4 encoding gene was absent.

The Cuban Institute for Swine Research recently reported that gastroenteric diseases cause 31% and 37% of mortality in suckling and weaned piglets, respectively (Cabrera and García, 2009). In suckling piglets, another causes of death, most of them closely related with diarrhea, such as crushing by the sow (14%), malnutrition (8%), congenital diseases (e.g. myoclonia; 6%), low weight at birth (4%), and hypoglycemia (3%) also require special attention. In weaned piglets, respiratory diseases (9%), malnutrition (5%), and classic swine fever (5%) are common causes of death (GRUPOR, 2008; Cabrera and García, 2009). Therefore, in 2008 the vice-director of the Cuban National Institute of Veterinary Medicine recommended an improvement on swine management and on the specific diagnosis of enteric diseases in order to decrease mortality by diarrhea in young pigs (Ricardo, 2008).

Nowadays, policies of the Cuban Ministry of Agriculture are to improve and increase swine production as pork is the most consumed meat in the country. In 2008 the Cuban swine herd was estimated to be 1,878,600 pigs (ONE, 2011), with the national company GRUPOR ruling the swine production (Fig. 1; Rico, 2005).

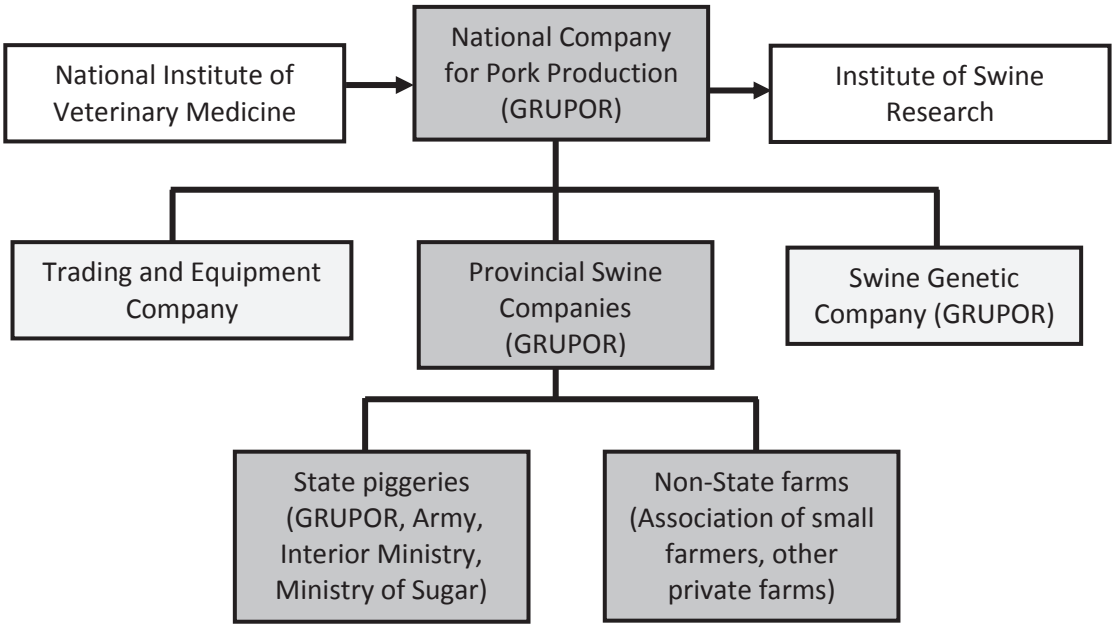
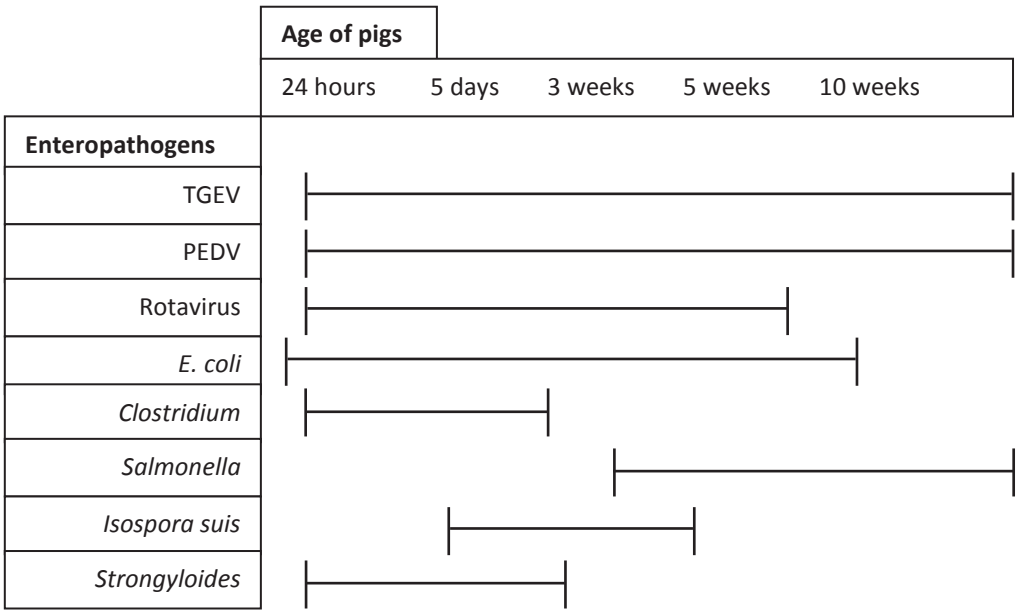


Figure 1. Organization of the swine production in Cuba.

Table 1. Range of age at which the most common swine enteropathogens can cause disease.



Multi-infectious etiology of diarrhea in young pigs

The clinical pictures of porcine pre- or post-weaning diarrhea often do not point to a specific etiology because of variations in clinical signs and epidemiology that can be produced by the effect of one or more disease agents. A control program of diarrhea could fail if one agent playing a primary role in the pathogenesis remains sub-diagnosed. An explosive onset and rapid spread of diarrhea is usually associated with a viral etiology. An insidious onset, slow spread, and gradual increase in severity over time tend to be seen with bacterial or parasitic diseases (Straw *et al.,* 2006).

A useful tool for veterinary practitioners regarding presumptive diagnosis of infectious diarrhea is the age at which piglets are affected. But this is rather an indicator of which pathogens could be responsible, and is not as precise as a specific etiologic identification test (Table 1; Straw et al., 2006).

The prevalence and repetitiveness of combined infections of enteropathogens reported worldwide lead to the introduction of the term ¨*mixed-type*¨ during epidemiological analysis of diarrhea. However, most prevalence studies on swine enteropathogens focused on single pathogens (Quilez *et al*., 1996; Barreiros *et al*., 2003) and checking for a multi-infectious etiology has often been neglected (Chae *et al*., 2000). Studies aimed at the differential identification of gastroenteric infections have displayed swine diarrhea as a complex syndrome in which several pathogens can be associated (Table 3), explaining a difficult control of pre- and post-weaning diarrhea in a piggery where enteropathogen identification has not been well performed. A few surveys undertaken to differentially identify enteric pathogens in suckling and weaned pigs with diarrhea indicate a high diversity of mixed infections which might lead to or complicate diarrheal outbreaks in a piggery (Table 4). However, worldwide available data regarding combined infections of enteropathogens remain difficult to compare as different tests were used for the same pathogen or diagnoses did not cover all potential enteropathogens.

The main characteristics for differential diagnosis of swine gastroenteric infections were summarized in Table 2 (adapted from Thomson, 2006).

Table 2. Insights for the differentiation of some common enteric diseases of young pigs.

Pathogens	Age	Signs	Gross lesions	Histological lesions	Laboratory methods
TGEV and PEDV	All ages	Watery diarrhea. Rapid dehydration. Vomiting	Thin-walled pale intestine, sparse content	Severe villous atrophy	PCR, IHC, ISH, or IF of intestinal contents. Serology
Rotavirus	1 day to 7 weeks old. Most frequent at 2-3 weeks	Watery-pasty diarrhea, sub-clinical. Varying dehydration	Fluid ingesta, pale intestines. Sparse stomach contents	Moderate villous atrophy	Virus detection: PAGE, PCR, ELISA, Tissue IHC
E. coli (ETEC, EPEC)	Neonatal: 1-4 days. Post-weaning: 1-3 weeks after weaning.	Watery, yellowish diarrhea. Sudden death. Dehydration.	Fluid ingesta, small intestinal congestion, watery content. Stomach full of milk.	Mucosal congestion, edema. Bacterial attachment to gut epithelia.	Culture, serotypes of isolates, PCR. Tissue IHC. Agglutination test.
C. perfringens type C	1-14 days (rarely older)	Hemorrhagic watery diarrhea. Sudden death	Hemorrhagic intestines, mucosal necrosis	Mucosal necrosis, associated with $Gram^+$ rods	*C. perfringens* toxins ELISA's on intestinal content. Histopathology
Salmonella spp.	All ages after weaning	Variable, watery muco-hemorrhagy. Most infections subclinical	Fibrinous or hemorrhagic, ulcers, intestinal lesions	Ulcers, neutrophil infiltration, fibrinous thrombi	Culture, serotyping, phage type. Antibody detection.
Cryptosporidium spp.	3 days to weaning	Mild-moderate yellowish diarrhea. Varying degrees of dehydration	Fluid ingesta	None or mild villous atrophy. Oocists in the epithelium	Mucosal smear for cryptosporidial oocysts. Histopathology
I. suis	5-21 days	Watery/yellowish diarrhea. Dehydration	Fluid ingesta, necrosis of intestinal mucosa	Villous atrophy, fibronecrotic enteritis, intracellular coccidians.	Stained mucosal smear. Histopathology. Identification of *Coccidia*

Table 3. Reports on differential identification and occurrence of enteropathogens in suckling (S) and weaned (W) pigs suffering from diarrhea worldwide.

Pigs	Pathogenic viruses (%)					Pathogenic bacteria (%)				
	Rotavirus	Coronavirus	Sapovirus	Adenovirus	PRRSV	ETEC	*Salmonella*	*C. perfringens*	*C. difficile**	*E. durans*
S	8	59.2 (TGEV)	NT	0.53	NT	19.6	NI	0.40 (type C)	NT	NT
	17	NT	NT	NT	NT	18.2	NT	NT	NT	NT
	5.1	3.4	NT	NT	NT	7.1	NI	NT	NT	NT
	42	6 (TGEV)	NT	NT	15	9**	NI	6^{NE}	55	12
	69.3	0	5.3	NT	NT	13.3	0	0 (type C)	NT	NT
	20.7	NT	NT	NT	NT	9.9	NT	0 (type C)	NT	NT
	13.3	NT	NT	NT	NT	10	NT	43.3 (type A), 0 (C)	23.3	NT
W	0	51.6	NT	NT	NT	42.7	NI	NT	NT	NT
	59.3	0	16.2	NT	NT	54.7	0	0 (type C)	NT	NT
S&W	4	13.4	NT	NT	NT	17.6	NI	NT	NT	NT

Pigs	Parasites (%)					Negatives (%)	Country	Reference
	I. suis	*Eimeria* spp.	*C. parvum*	*B. coli*	Helminths			
S	15.3	0	NT	NT	NT	9.2	Canada	Morin *et al.,* 1983
	53.8	0	NT	NT	NT	NI	Australia	Driesen *et al.,* 1993
	28.8	NI	NI	NI	0	NI	Germany	Wieler *et al.,* 2001
	NT	NT	NT	NT	NT	NI	U.S.A.	Yaeger *et al.,* 2002
	18.7 (*Coccidia*)		0	NI	NI	17	Japan	Katsuda *et al.,* 2006
	18.8 (*Coccidia*)		NT	6.9	NT	15.8	Romania	Costinar *et al.,* 2010
	0	NI	NI	NI	NI	NI	Brazil	Cruz *et al.,* 2010
W	1.2	NI	NI	NI	0	NI	Germany	Wieler *et al.,* 2001
	18.6 (*Coccidia*)		16.2	NI	NI	9.5	Japan	Katsuda *et al.,* 2006
S&W	20.9	0.7	1.4	0.7	0	57.7	Germany	Wieler *et al.,* 2001

NT, not tested; NI, no information; *, toxin detection; **, no determination of virulence factors and serotypes; ^{NE}, necrotic enteritis.

Table 4. Occurrence (%) of enteric mixed infections in suckling (S) and weaned (W) pigs with diarrhea reported worldwide.

¨Mixed types¨	Tested	%	S/W	Country	Reference
Rotavirus + PEDV	157	45.2	S	South Korea	Song *et al.,* 2006
	98	9.1		Czech Republic	Czanderlova *et al.,* 2010
Rotavirus + TGEV	749	0.8	S	Canada	Morin *et al.,* 1983
	98	4.5		Czech Republic	Czanderlova *et al.,* 2010
Rotavirus + TGEV + PEDV	98	22.7	S	Czech Republic	Czanderlova *et al.,* 2010
Rotavirus + PRRSV	100	1	S	U.S.A.	Yaeger *et al.,* 2002
Rotavirus + Sapovirus	153	1.3	S	Japan	Katsuda *et al.,* 2006
	116	1.7	W		
Rotavirus + Sapovirus + ETEC	153	2	S		
	116	2.6	W		
Rotavirus + TGEV + ETEC	749	0.66	S	Canada	Morin *et al.,* 1983
PRRSV + *Clostridium difficile*	100	3	S	U.S.A.	Yaeger *et al.,* 2002
Rotavirus + *Clostridium difficile*	100	6			
TGEV + ETEC	749	4.3	S	Canada	Morin *et al.,* 1983
Rotavirus + ETEC	749	1.07	S	Canada	Morin *et al.,* 1983
	1054	1.0		Australia	Driesen *et al.,* 1993
	100	1.0		U.S.A.	Yaeger *et al.,* 2002
	153	6.5		Japan	Katsuda *et al.,* 2006
	116	19.0	W		
Rotavirus + Sapovirus + ETEC + *Coccidia*	116	6.0	W	Japan	Katsuda *et al.,* 2006
Rotavirus + Sapovirus + ETEC + *C. parvum*	116	0.9	W		
Sapovirus + ETEC + *C. parvum*	116	0.9	W		
Rotavirus + Sapovirus + *Coccidia*	153	0.7	S		
	116	0.9	W		
Rotavirus + Sapovirus + *C. parvum*	116	0.9	W		
Rotavirus + TGEV + *Coccidia*	749	0.13	S	Canada	Morin *et al.,* 1983
Rotavirus + ETEC + *Coccidia*	1054	0.6	S	Australia	Driesen *et al.,* 1993
	101	2.9		Romania	Costinar *et al.,* 2010
	153	1.3		Japan	Katsuda *et al.,* 2006
	116	3.4	W		
Rotavirus + ETEC + *Coccidia* + *C. parvum*	116	2.6	W	Japan	Katsuda *et al.,* 2006
Rotavirus + ETEC + *Coccidia* + *B. coli*	101	0.9	S	Romania	Costinar *et al.,* 2010
Rotavirus + *Coccidia*	9*	55.6	S	U.S.A.	Roberts and Walker, 1982
	749	1.2		Canada	Morin *et al.,* 1983
	1054	6.7		Australia	Driesen *et al.,* 1993
	101	8.9		Romania	Costinar *et al.,* 2010
	153	7.2		Japan	Katsuda *et al.,* 2006
	116	2.6	W		
Rotavirus + *Coccidia* + *B. coli*	101	6.9	S	Romania	Costinar *et al.,* 2010
Rotavirus + *C. parvum*	116	2.6	W	Japan	Katsuda *et al.,* 2006
Sapovirus + *Coccidia*	153	0.7	S		
TGEV + *Coccidia*	749	2.8	S	Canada	Morin *et al.,* 1983
ETEC + *Clostridium difficile*	100	1.0	S	U.S.A.	Yaeger *et al.,* 2002

Table 4 (continuation). Occurrence (%) of enteric mixed infections in suckling (S) and weaned (W) pigs with diarrhea reported worldwide.

¨Mixed types¨	Tested	%	S/W	Country	Reference
ETEC + *Coccidia*	749	0.8	S	Canada	Morin *et al.,* 1983
	1054	10.7		Australia	Driesen *et al.,* 1993
	153	2.6		Japan	Katsuda *et al*., 2006
	116	0.9	W		
ETEC + *C. parvum*	116	2.6	W	Japan	Katsuda *et al.,* 2006
Occurrence of multiple infections	749	11.7	S	Canada	Morin *et al.,* 1983
	1054	19.1		Australia	Driesen *et al.,* 1993
	100	12		U.S.A.	Yaeger *et al.,* 2002
	101	27.7		Romania	Costinar *et al.,* 2010
	153	22.2		Japan	Katsuda *et al.,* 2006
	116	47.4	W		
	92	9.0		Hungary	Nagy *et al.,* 1996
	287	9.1	S&W	Germany	Wieler *et al.,* 2001

In this table and in references to this table *Coccidia* refers to *I. suis* and or *Eimeria* spp.

As shown in Table 4, combined infections of Rotavirus-ETEC, Rotavirus-*Coccidia*, ETEC-*Coccidia*, and Rotavirus-ETEC-*Coccidia* have been commonly reported. Despite a low occurrence, the associations Rotavirus-ETEC-*Coccidia*-*C. parvum*, TGEV-Rotavirus-ETEC, and TGEV-Rotavirus-*Coccidia*, are important to consider because of the proven pathogenic effect of the involved agents.

Katsuda *et al*. (2006) in Japan and Costinar *et al*. (2010) in Romania reported that 22.2% and 27.7% of suckling diarrheic piglets, respectively, carried mixed infections. Therefore, mixed infections have to be accurately identified during diarrhea outbreaks to plan and implement an efficient disease surveillance, prevention and control in every piggery, geographic area, or management system.

Knowledge on the role of individual enteropathogens during pathogenesis and course of combined enteric infections is limited. Practically, no controlled experiments have been designed to study the interaction between the diverse enteropathogens; however, interesting findings have been reported and discussed by some authors:

- Baba and Gaafar (1985) demonstrated that piglets which have been infected with *S. typhimurium* and subsequently with *I. suis*, showed significantly smaller ($p<0.05$) *S. typhimurium* counts in the feces, caecal contents and mesenteric lymph nodes than those infected with *S. typhimurium* alone. They mentioned as possible causes the cleansing effect and low intestinal pH due to coccidial diarrhea, but the exact mechanism remains to be clarified.

- Lecce *et al. (*1982) suspected that rotavirus provokes a damage of the epithelium that can alter binding sites on enterocytes, favoring gut colonization by ETEC. Similar observations were made by Melin *et al. (*2004).
- Necropsy seventeen days post-*Ascaris* infection revealed lower hepatic fibrosis and a lesser degree of liver eosinophilia in *Ascaris* infected pigs pre-inoculated with *Salmonella* than those receiving *Ascaris* inoculation only (Wade and Gaafar, 1981). Similar results were seen in pigs recovered from TGE and later infected with *Ascaris* (Gaafar *et al.,* 1973).
- Janke *et al*. (1988) concluded that rotaviral infection in colostrum-deprived piglets is inhibited by enteroviruses infection.
- Choi *et al. (*2003), after using plant lectins for studying the histochemistry of the jejunal mucosa of pigs infected with *I. suis,* argue that variations on the glycoconjugates composition due to isosporosis could favor *E. coli* colonization.
- Enemark *et al. (*2003a) found that mixed infections of rotavirus and *Cryptosporidium* cause a dramatic aggravation of diarrhea and clinical signs; piglets mono-infected with Rotavirus or *Cryptosporidium* showed no or very mild clinical signs of illness.
- *C. perfringens* type C may colonize lesions initiated by *Coccidia*, rotavirus, TGEV and PEDV (Songer and Uzal, 2005).
- Zintl *et al. (*2007) found that *Cryptosporidium* infections are not commonly combined with *Salmonella* infections in pigs.
- Jung *et al. (*2008) proposed that the severe diarrhea in PEDV-rotavirus A co-infected piglets may be more associated with the immunity level of the host rather than to any synergistic effect of rotavirus on PEDV enteritis. They suggested that concurrent infection with porcine rotavirus A does not synergistically enhance intestinal villous atrophy.
- Kim *et al. (*2010a) reported that 124/182 (68%) rotavirus A infections were mixed with other enteric pathogens.

Major enteropathogens causing diarrhea in young pigs

Escherichia coli

E. coli are an important cause of diarrhea in suckling and recently weaned pigs, and are responsible for significant economical losses worldwide. Most porcine ETEC and VTEC strains produce fimbriae, which enable them to colonize the epithelial surface of the porcine small intestine. Pathology is the result of the production of toxins. ETEC strains produce heat-stable (STa or STb) or heat-labile (LT) which induce fluid loss resulting in diarrhea (Nataro and Kaper, 1998).

VTEC produce the verocytotoxin STx2e which is responsible for edema disease. Some *E. coli* strains can produce both enterotoxins and the verocytotoxin STx2e which is responsible for edema disease, and are appropriately referred to as ETEC/VTEC (Blanco *et al.,* 2006).

Attachment to specific receptors is essential for colonization and pathogenesis. Fimbriae mediate the attachment of *E. coli* to the intestinal mucosal surface. F4 and F18 fimbriae have been commonly identified in *E. coli* associated with PWD. But, whereas F18 fimbriae are almost exclusively associated with PWD, F4 fimbriae are also a predominant colonization factor implicated in neonatal diarrhea. Of F4, 3 serotypes have been described, namely F4ab, F4ac and F4ad, of which F4ac is the most prevalent (Choi and Chae, 1999). Of F18, two serotypes have been identified: F18ab and F18ac. F18ab is more expressed by VTEC strains and F18ac by ETEC strains. Other fimbriae such as F5, F6, and F41 are mainly expressed by porcine ETEC isolated from newborn piglets with diarrhea. Nevertheless, F4 and to a lesser extent F18, continue to be the major fimbrial antigen types in ETEC and VTEC identified in diagnostic laboratories in the U.S.A. (Moon *et al.,* 1999), China (Cheng *et al.,* 2006), Brazil (Vidotto *et al.,* 2009), Zimbabwe (Madoroba *et al.,* 2009), and Vietnam (Oanh *et al.,* 2010).

Some candidate receptors have been suggested for $F4^+$ *E. coli* in the gut epithelium of pigs (F4R). Erickson *et al.* (1992; 1994) proposed the intestinal mucin-like sialoglycoproteins (F4acR; IMTGPs), Grange and Mouricout (1996) reported an intestinal transferrin (F4abR) and Grange *et al. (*1999) an intestinal glycosphingolipid (F4adR). Recently, Rasschaert (2008) identified porcine aminopeptidase-N (pAPN) as a receptor for F4ac. Looking at binding to glycosphingolipids, Coddens *et al*. (2011) found that F4ab binds galactosylceramide, sulfatide, sulf-lactosylceramide and globotriaosylceramide present in epithelial cells of the porcine intestine, whereas F4ac only binds galactosylceramide.

Not all pigs have receptors for adhesion of $F4^+$ and $F18^+$ *E. coli.* Some pigs are receptor negative. Testing *in vitro* adhesion of the three F4 variants (ab, ac, and ad) to brush border membranes of enterocytes, isolated enterocytes or isolated villi, six phenotypes could be identified: phenotype A binds all three F4 variants, phenotype B F4ab and F4ac, phenotype C F4ab and F4ad, phenotype D F4ad, phenotype E none of the variants, and phenotype F F4ab only. Based on that classification, prevalence studies have been done in different swine herds: in Midwestern United States and The Netherlands 30% and 50% of pigs showed the phenotype E, respectively (Sellwood *et al.,* 1975, Baker *et al.,* 1997; Bijlsma *et al.,* 1985). Conversely, 80% of the Belgian pigs were classified in the F4 susceptible phenotype A, and only 4% in the resistant phenotype E (Cox and Houvenaghel, 1987).

The F4ab/ac receptor loci are closely linked and linked to the transferrin locus on chromosome 13. They behave as a single autosomal dominant gene (Guérin *et al.,* 1993). Python *et al. (*2002)

concluded that the receptor for F4ac binds F4ab bacteria as well, and that it is controlled by one gene localized between S0068 and Sw1030 on chromosome 13. Jørgensen *et al.* (2004) detected that a mutation G→C in intron 7 of *mucin 4* gene was strongly associated with the ETEC F4ab/ac adhesive phenotype. They developed a PCR-RFLP which detected polymorphisms in a *mucin 4* gene fragment by its digestion with *Xba*I enzyme, and allows genotyping pigs for resistance or susceptibility to adhesion of *E. coli* mediated by F4ab/ac fimbriae. Three different pig genotypes can be observed: resistant (no adhesion) homozygote carrying indigestible alleles (RR) and susceptible heterozygote (SR) as well as homozygote (SS). Conversely, Rasschaert *et al. (*2007) could not confirm this good correlation when comparing results of the DNA-based marker test with results of the *in vitro* villous adhesion assay. A reason for the difference in both studies could be the use of a different *in vitro* adhesion assay as Jørgensen *et al. (*2004) used single enterocytes whereas Rasschaert *et al. (*2007) used isolated villi.

Looking at binding of F18 to glycosphingolipids, Coddens *et al. (*2009) could identify the receptors for F18. They observed a high specific interaction of F18 *E. coli* with glycosphingolipids having blood group A/B/H determinants on type 1 core chains, as well as the blood group A type 4 heptaglycosylceramide.

The *in vitro* villous adhesion tests also allow discrimination between $F18R^+$ and $F18R^-$ pigs. Absence or presence of the F18R is genetically determined and susceptibility being dominant over resistance (Bertschinger *et al.,* 1993). The gene controlling expression of the F18R was mapped to the halothane linkage group on pig chromosome 6 (Meijerink *et al.,* 1997). This locus contained two candidate genes, *FUT1* and *FUT2*, both encoding α2-fucosyltransferases. Expression analysis of these two genes in the porcine small intestine revealed that only the *FUT1* gene was expressed in all examined pigs (Meijerink *et al.,* 2000). This gene is localized in chromosome 6q11 and the enzyme alpha (1,2)-fucosyltransferase 1 transfers fucose to lipid, carbohydrate, and protein backbones present in the intestine. Sequencing of this gene showed a polymorphism (G or A) at nucleotide 307 resulting in an amino acid substitution at position 103 (Ala/Thr) of the enzyme. Presence of the A nucleotide on both alleles (*FUT1A/A* genotype) led to significantly reduced activity of the enzyme corresponding to the F18-fimbriated *E. coli* resistant genotype, whereas susceptible pigs had either the heterozygous *FUT1G/A* or the homozygous *FUT1G/G* genotype. These findings have led to the development of a PCR-RFLP using *Cfo*I enzyme that allows the classification of pigs into susceptible or resistant to $F18^+$ *E. coli* adhesion (Meijerink *et al.,* 1997).

Frydendahl *et al. (*2003) found a high correlation between $F18R^+$ genotypes and susceptibility to $F18^+$ *E. coli*; however, pigs carrying the resistant F18R genotype were not entirely protected against intestinal colonization. Coddens *et al. (*2007) also found a significant positive but weak correlation

(r=0.307, p<0.05) between the *FUT1* genotype and the F18R phenotype. Indeed they reported a weak adhesion of $F18^+$ *E. coli* to the villous brush border in some genetically $F18R^-$ pigs and absence of adhesion in some $F18R^+$ pigs.

Heat-labile enterotoxins (LT) produced by ETEC have a high molecular weight (86 kDa) and two major serogroups, which do not cross-react immunologically, have been identified: LT-I and LT-II. Only LT-I is neutralized by anti-cholera toxin. LT produced by porcine *E. coli* (LTp) belongs to the LT-I group. The toxin complex is constituted by one A subunit (biologically active) and five B subunits, which bind mainly to gangliosides and some intestinal glycoproteins (Teneberg *et al.,* 1994). The A subunit stimulates the production of c-AMP by activating adenylate cyclase located on the basolateral membrane of polarized intestinal epithelial cells, resulting in a secretory diarrhea due to a decrease in Na^+ absorption by villus cells and activation of Cl^- secretion by crypt cells. As for cholera toxin, additional mechanisms influencing intestinal motility and ion secretion have been attributed to the mode of action of LT, i.e. increase of prostaglandin-E release, modeling of the enteric nervous system via vasoactive intestinal polypeptides and a mild intestinal inflammatory response involving interleukin-6 (Sears and Kaper, 1996; Nataro and Kaper, 1998; Fairbrother and Gyles, 2006).

The heat-stable enterotoxins (STa and STb) are antigenically and genetically unrelated monomeric toxins that contain multiple cysteine residues, whose disulfide bonds account for the heat stability. STa is a small (2 kDa), non-immunogenic, protein most effective in piglets younger than 2 weeks of age. STa acts on guanylate cyclase so increasing the concentration of c-GMP in epithelial cells (Vaandrager *et al.,* 1994), leading to inhibition of Na^+ absorption and stimulation of Cl^- secretion with water passively following resulting in a secretory diarrhea. Two variants have been described for STa: STp has been identified in porcine, bovine and human ETEC, while STh is only produced by ETEC strains infecting humans (Fairbrother and Gyles, 2006). STb is also a small (5 kDa), poorly immunogenic, protein that strongly binds to acidic glycosphingolipids, including sulfatide (or 3'-sulfogalactosylceramide) and several gangliosides (Dubreuil, 1997; Rousset *et al.,* 1998).

Table 5 shows results of diverse surveys aimed at detecting virulence encoding genes in *E. coli* isolated from feces of young pigs worldwide.

In addition to the natural existence of diverse *E. coli* virulence factors which lead host colonization and disease, the increase in antimicrobial resistance which is occurring in ETEC and VTEC strains, make swine colibacillosis difficult to control. Due to the young age in which piglets become infected with these pathogens, many breeders easily and systematically administer antibiotics resulting in a high selection pressure towards resistance. Boerlin *et al*. (2005), Travis *et al*. (2006) and Wang *et al*. (2010) reported clear associations between antibiotic resistance and virulence genes in swine pathogenic *E. coli*. For instance, genes coding for Sta/STb and tetracycline

resistance were found to be co-located on a self-conjugative plasmid (pTC, 120-kb) which is widely distributed among porcine ETEC (Olasz *et al*., 2005).

As good management, hygiene and vaccination in swine farms, also antibiotics have helped in preventing and controlling swine colibacillosis, but it also provoked the appearance and selection of resistant and multidrug-resistant *E. coli* which tend to persist in time and space (Maidhof *et al*., 2002; Blake *et al*., 2003; Moredo *et al*. 2007; Dewulf *et al*., 2007; Akwar *et al.* 2008; Vieira *et al*., 2009; Bibbal *et al*., 2009). In New Zealand, Nulsen *et al*. (2008) found a higher antibiotic resistance to tetracycline (60% v/s 5%), streptomycin (25% v/s 3%), and cotrimoxazole (11% v/s 0%) in *E. coli* isolated on conventional farms (where is a higher antibiotic pressure) than on organic farms. Maynard *et al.* (2003) concluded that the genes behind phenotypic antibiotic resistance are not static and their prevalence is determined by various selection forces such as the use of specific antimicrobials.

Investigation of virulence factors, antimicrobial resistance, and genetic profiles are essential to deeply study the epidemiology of ETEC associated with swine diarrhea. ETEC carrying the same virulence profiles and with similar antibiogram sensitivity are likely to show a highly similar pulsotype by PAGE (Lee *et al*., 2009; Bibbal *et al*., 2009). Such mixed studies are becoming an important epidemiological tool. Thorsteinsdottir *et al*. (2010) found the same resistance profile and pulsotype among *E. coli* isolated from broiler meat and slaughterhouse workers. They stated that isolates sharing the same genetic profile and resistance patterns can arise on different farms. Conversely, Rosengren *et al*. (2009) and Smith *et al.* (2010) did not find clear associations between antimicrobial resistance and virulence profile in *E. coli* isolated from healthy versus diseased pigs. Surveillance studies of swine colibacillosis should be applied to every geographic area or even at farm or production system level as a high variety of antibiotics, resistance genes, virulence genes, and their co-location onto conjugative plasmids or pathogenicity islands in *E. coli* lead to diverse associations or clones (Hendriksen *et al.*, 2008; Harada *et al.*, 2008; Wang *et al*., 2010; Smith *et al*., 2010). Additionally, the co-selection of *E. coli* resistant to some antibiotics (i.e. kanamycin) by the use of other antibiotics (i.e. tetracycline) is contributing to increase antibiotic resistance in swine farms. Therefore risk assessment has to be performed for every antibiotic or every chemical group in order to better police the selection and persistence of resistant *E. coli* (Harada *et al.*, 2008).

Table 5. Recent studies reporting virulence encoding genes in *E. coli* isolated from feces of young pigs worldwide.

Syndrome & age group	n	% from tested isolates											Country/Reference
		VF+	F4	F5	F6	F41	F18	Int	STa	STb	LT	STx2e	EAST1	
ND														
(<21 d.)	200	63	33.5	10.5	0	0	0	NT	58	52.5	35	NT	NT	Vietnam/Do *et al.,* 2006
(<30 d.)	13	46.2_F, 100_T	0	0	23	0	23	NT	53.8	61.5	7.7	7.7	NT	Cuba/Blanco *et al.,* 2006
(<14 d.)	220	56_F, 74_T	38	3	3	3	9	3	13	49	42	4	65	Slovakia/Vu-Khac *et al.,* 2007
Healthy	30	17_F, 26.7_T	0	0	0	0	0	17	0	0	0	0	26	
(4-21 d.)	196	32_T	9.7	7.7	0.5	7.7	8.8	NT	9.2	15.8	16.3	8.8	NT	Zimbabwe/Madoroba *et al.,* 2009
ND&PWD														
(0-35 d.)	83	79.3_F, 90_T	45.8	18.4	26.3	5.3	26.3	10.5	80.7	44.6	45.8	15.7	NT	Japan/Katsuda *et al.,* 2006
(2-72 d.)	562	34	2.3	2	2.5	2.8	5.3	7.1	7.3	4.8	7.8	5.2	13.9	South Korea/Kim *et al.,* 2010b
PWD														
(4-6 w.)	372	29_F, 80.1_T	19.1^A	2.1^A	1.1^A	2.9^A	2.7	NT	12.4	4.3	57.3	20.4	NT	Poland/Osek, 1999
Healthy	46	3.5_F, 8.7_T	0^A	4.3^A	0^A	0^A	2.2	NT	2.2	0	2.2	4.3	NT	
(4-10 w)	215	50.2_F, 7.2_T	9.8^A	10.7^A	15.8^A	9.8^A	25.6^A	NT	74.4	14	2.3	8.8	NT	China/Chen *et al.,* 2004
(>30 d.)	23	82.6_F, 100_T	0	0	0	0	82.6	NT	65.2	73.9	4.3	78.2	NT	Cuba/Blanco *et al.,* 2006
-	101	60_F, 77_T	19	0.9	5	0.9	35	0.9	26	46	20	5	64	Slovakia/Vu-Khac *et al.,* 2006
-	304	58_F	37.1	0.3	0	0.3	19.7	0.7^D	15.8	41.8	33.2	9.9	20	U.S.A./Zhang *et al.,* 2007
-	100	100	44	30	25	32	38	NT	40	47	71	3	NT	Brazil/Vidotto *et al.,* 2009
PWD&ED														
-	230	40.9	10	1.7	4.3	0.8	18.3	NT	27.5	15.2	8.7	15.2	NT	South Korea/Kwon *et al.,* 2002
(4-8 w.)	219	85.4_F, 87.2_T	44.7	0	0.9	0	39.3	1.4	26.5	7.6	61.6	16.4	65.8	Denmark/Frydendahl, 2002
-	240	100	3.7	0	0	0	26.2	28.3	14.5	9.1	10.8	35	NT	China/Cheng *et al.,* 2006

ND, neonatal diarrhea; PWD, post-weaning diarrhea; ED, edema disease; n, tested *E. coli* isolates; VF, virulence factor; $_{T \text{ and } F}$, positive for at least one of the tested toxin or adhesin genes respectively; ^{A}, identified by the agglutination test; ^{D}, Zhang *et al. (*2007) also reported AIDA-I (15.5%) and PAA (34.5%); NT, not tested; d., days old; w., weeks old.

Clostridium perfringens

C. perfringens is a Gram-positive anaerobic bacterium that is able to form spores. It is widespread in the environment (e.g. in soil and sewage) and is commonly found in the intestine of animals. *C. perfringens* strains are classified into five toxinotypes (A, B, C, D, and E) according to the production of alfa (α), beta (β), epsilon (ε) and or iota (ι) toxins which are crucial in the pathogenesis of clostridiosis (Petit *et al.,* 1999); besides, they can also produce a pore-forming enterotoxin called *C. perfringens* enterotoxin (CPE; McClane, 1996) and a cytotoxic β2-toxin (Garmory *et al*., 2000) which are usually tested for subtyping.

Alfa and β-toxigenic strains have been associated with swine diarrhea worldwide (Morin *et al.*, 1983; Niestrath *et al.,* 2002; Yaeger *et al.*, 2002; Das *et al.*, 2009; Cruz *et al.,* 2010).

C. perfringens type-C produce α and β toxins and cause hemorrhagic, often fatal, necrotic enteritis in young piglets. The disease is most frequent in 3-day-old piglets, but it can appear in the first 12 hours of life (Songer and Meer, 1996; Songer and Uzal, 2005). This strain is rarely found in the intestine of healthy piglets. It is important to remark that piglet-piglet transmission occurs, and spores persist in the environment as they are resistant to heat, disinfectants, and ultraviolet light. The main source of infection in a piggery is the intestine of the sow (Songer, 1996). Type C clostridiosis can occur epidemically in non-vaccinated herds, and prevalence in affected litters can reach 100% with mortality close to 100% (Songer and Uzal, 2005).

The basis of the pathogenicity of *C. perfringens* type A strains (α-toxigenic) frequently isolated from pigs with enteritis has not been clearly established but necrotic intestinal lesions have been experimentally induced by inoculation of *C. perfringens* type A culture supernatant (Songer, 1996). The enteropathogenicity of these strains might result from high levels of α-toxin production, from molecular variants that are more stable to protease digestion or are more active, from different host sensitivity to α-toxin (Ginter *et al.,* 1996), or due to the side production of β2-toxin (Garmory *et al*., 2000; Hendriksen *et al*., 2006) or the *C. perfringens* enterotoxin.

The α-toxin is a phospholipase C sphingomyelinase that hydrolyzes phospholipids (e.g. lecithin) and promotes membrane disorganization, resulting in blood vessel contraction, increased vascular permeability, platelet aggregation and myocardial dysfunction, all of which contribute to local and systemic clinical manifestations (Bunting *et al.,* 1997; Naylor *et al.,* 1998). The β1- and β2-toxins induce hemorrhagic necrosis of the intestinal mucosa (Gibert *et al.,* 1997). Although these toxins are cytotoxic (Gibert *et al.,* 1997), their mode of action has not yet been completely elucidated. The fact that β1-toxin displays a significant homology at the amino acid level with α-toxin, and leucocidin of *Staphylococcus*

aureus which form multimers and pores in eukaryotic cell membranes, suggests that β1-toxin has a similar mode of action (Hunter *et al.,* 1993). Gurtner *et al.* (2010) confirmed that β1-toxin causes disruption of the actin cytoskeleton of endothelial cells. Also, *C. perfringens* secretes a variety of hydrolytic enzymes that degrade extracellular substrates and components resulting from cell lysis. It is possible that these enzymes act synergistically with membrane-damaging toxins during cell disruption (Petit *et al.,* 1999). Zeng *et al*. (2011) developed and recommended the application of recombinant fusion toxoids as good vaccine candidate against the α, β1, and β2 clostridial toxins.

Testing for *C. perfringens* toxins by enzyme-linked immunosorbent assay (ELISA) is a reliable test (Naylor *et al.,* 1997; Niestrath *et al.,* 2002), i.e. the commercially available Bio-X ELISA kit (Bio-X Diagnostics, Marche-en-Famenne, Belgium) that detects the α-, β- and ε-toxins in intestinal contents or culture supernatants. Genotyping of *C. perfringens* has simplified the routine diagnosis. Multiplex-PCR for detecting fragments of genes encoding toxins enables the diagnostician to screen larger numbers of samples with higher accuracy and greatly reduces the amount of false-negative results (Das *et al*., 2009; Baker *et al*., 2010).

In summary, the clinics and the differential diagnosis with other enteropathogens is very important to consider when implicating *C. perfringens* as primary agent. Toxinotype A is widespread in the intestines of pigs worldwide (Table 6) and its association with diarrhea has to be carefully analyzed in every outbreak. Type C strains frequently cause hemorrhagic necrotic enteritis in young pigs (Petit *et al.,* 1999).

Table 6. Reports of surveys assessing association of *C. perfringens* with swine diarrhea worldwide.

Age groups	Tested	%	Associated toxins						Assay	Country/Reference
			α	β1	β2	ε	CPE	ι		
1-15 d.	749	0.4	+[NE]	+[NE]					Clinics, histopathology general bacteriologic procedures	Canada/Morin *et al.,* 1983
1-3 d.	13	100[M]	+						Based on anamnesis, differential diagnose, histopathology, general bacteriologic procedures, inoculation bioassay and reverse passive latex agglutination assay	U.S.A./Collins *et al.,* 1989
Suckers	33	87.9	+	-	+*	-	-	-	General bacteriologic procedures, PCR	U.S.A./Garmory *et al*., 2000
	33	12	+	+	+	-	-	-		
	7[ND]	0	-	-	-	-	-	-		
Suckers	10[DN]	50[I.S.]	+	-	NT	-	NT	NT	DAS-ELISA	Germany/Niestrath *et al.,* 2002
1-7 d.	100	6	+[C]	+[C]					Necrotizing intestinal lesions in association with large, gram-positive bacilli lining necrotic villus remnants. Dense grow of *C. perfringens*	U.S.A./Yaeger *et al.,* 2002
Suckers	220[A]	90.9	+	-	+	-	-	-	PCR (during this survey *C.p.* isolates were only tested for cpb2 gene which codes for β2 toxin)	U.S.A./Bueschel *et al*., 2003
	36[C]	97.2	+	+	+	-	-	-		
	9[C.p. ND]	11.1	-	-	+	-	-	-		
Suckers	153	0	-	-	NT	-	NT	-	General bacteriologic procedures, PCR	Japan/Katsuda *et al.,* 2006
Weaners	116	0	-	-	NT	-	NT	-		
12-14 m.	146	2.1[T]	+	-	+	-	-	-	General bacteriologic procedures, PCR	Malaysia/Das *et al.,* 2009
2-3 m.	96	2.1[T]	+	-	+	-	-	-		
1-7 d.	30	33.3	+	-	+	-	-	-	General bacteriologic procedures, PCR	Brazil/Cruz *et al.,* 2010
Suckers	333	89.8	+	-	NT	-	-	-	General bacteriologic procedures, PCR	U.S.A./Baker *et al*., 2010

[NE], based on findings of necrotic enteritis which is associated with *C. perfringens* C; [M], morbidity close to 100% and 13 piglets (8 sick and 5 healthy) were selected for *C. perfringens* study; [*], in 79**%** of *C. perfringens* A; [ND], non diarrheic control piglets, [I.S.], mixed with *I. suis,* and 1/5 positive piglets had necrotic enteritis; [C], *C. perfringens* type C; [A], *C. perfringens* type A; [C.p. ND], *C. perfringens* isolated from non diarrheic piglets; [T], *C. perfringens* was isolated from pigs which died after tetracycline resistant acute diarrhea; [DN], diarrheic and non-diarrheic pigs.

Transmissible gastroenteritis virus and porcine epidemic diarrhea virus

TGEV belongs to the *Coronaviridae* family. The virus contains a single-stranded genomic RNA and only one serotype is known (Kemeny, 1976). Three major structural proteins described for coronaviruses are the spike glycoprotein (S; 180–200 kDa), the membrane protein (M; 21–30 kDa), and the nucleoprotein (N; 45–50 kDa) (Spaan *et al.,* 1988). The S protein is the most interesting protein from an antigenic and immunogenic point of view (Delmas *et al.,* 1986; Jiménez *et al.,* 1986; Torres *et al.,* 1995). Four antigenic sites (C, B, D, and A) were mapped on the S protein starting from the N-terminal end and antibodies against them can be found in the serum of TGEV-infected pigs (Correa *et al.,* 1990). The spike glycoprotein initiates infection by binding to the enterocytes via pAPN, which has been identified as a coronavirus receptor (Delmas *et al.,* 1992; Hansen *et al.,* 1998). PEDV, another coronavirus, also binds specifically to pAPN and this binding can be inhibited by anti-pAPN antibodies (Oh *et al.,* 2003; Li *et al.,* 2007). TGEV shows also sialic acid binding activity, perhaps providing a second binding site that may account for the enteropathogenicity of the different strains (Schwegmann-Wessels *et al.,* 2002; Schwegmann-Wessels and Herrler, 2006).

Three swine coronaviruses are pathogenically or antigenically related: TGEV, PEDV, and porcine respiratory coronavirus (PRCV). TGEV and PRCV cross-react serologically and are very closely related only differing in a deletion mutation of 224-227 amino acids in the Spike protein S of PRCV in comparison with TGEV. PRCV cannot be distinguished from enteropathogenic strains of TGEV by a virus neutralization test (Callebaut *et al.,* 1988). Indeed, infection with PRCV induces the production of antibodies able to neutralize both TGEV and PRCV at the same titer (Pensaert *et al.,* 1986). Previous PRCV infections in piglets and or sows, or concurrent TGEV/PRCV infections influence and change the pathogenesis of TGEV, thereby reducing the severity of disease (Cox *et al*., 1993; Kim *et al.,* 2000). As a consequence, the presence of PRCV in Europe reduced the incidence and severity of epidemic TGE (Pensaert *et al.,* 1993). Therefore, a low prevalence (0.9%) of TGEV infection compared with PEDV in South Korea could be due to the high prevalence of PRCV (Chae *et al.,* 2000). Also in the United States and Japan, a decrease in TGE incidence has been reported in areas with a high prevalence of anti-PRCV antibodies (Yaeger *et al.,* 2002; Miyazaki *et al.,* 2010). In TGEV- and PRCV-seronegative herds, however, TGE remains a major cause of sickness and death in piglets (Barrera *et al.,* 2005; Saif and Sestak, 2006).

Characteristics of TGE acute form are a short incubation period, diarrhea, vomiting, and dehydration. Mortality approaches 100% in newborn piglets, but decreases with age. Sows infected shortly after parturition may be severely affected by diarrhea, hypogalactia, and agalactia (Djurickovic *et al.,* 1969; Saif and Sestak, 2006). The first outbreaks of TGE reported in February 2003 in Cuba were a classic

example of this form. On affected farms, 100% of recently farrowed sows and their litters had diarrhea. The clinical signs in newborns included very liquid and fetid, yellowish feces and vomiting, leading to serious dehydration. At the onset of the disease, sows showed a lack of appetite followed by vomiting and agalactia, but all recovered. The weaned and fattening pigs of these farms presented severe clinical signs, although only 8% lethality was reached. The disease spread rapidly to other farms over the island. In the Havana province, 15 outbreaks affecting 23.201 animals, caused 10.547 deaths, and 5.256 animals had to be slaughtered (Barrera *et al.,* 2005; IMV, 2003).

The clinical signs of TGE are usually milder when TGEV is introduced into seropositive farms, or when TGEV infects less susceptible animals, such as sows or finisher pigs in seronegative farms. Endemic TGE is limited to seropositive herds and diarrhea can occur in pigs from 6 days old until 2 weeks after weaning, and the mortality varies from 10-20% or even less. During this presentation TGE is difficult to differentiate from rotavirosis or enteric colibacillosis (Saif and Sestak, 2006). TGEV infection therefore occasionally goes undiagnosed. Risk for TGE was greater in herds with more than 50 breeding pigs than in smaller ones.($p<0.01$; Cubero *et al*., 1993).

Traditional methods to confirm TGEV infection are based on clinical signs, microscopic lesions, virus isolation, fluorescent antibody test, electron microscopy or immunohistochemistry, and an enzyme immunofiltration assay (Phillips and Westerman, 1991; Saif and Sestaf, 2006).

The appearance of the closely related PRCV complicated the diagnosis. However, the absence of 2 antigenic sites (B and C) in the S protein of PRCV as a consequence of the deletion of 224–227 amino acids, was the basis for its differentiation from TGEV (Rasschaert *et al.,* 1990) and a number of ELISA's based on MAb's directed against the antigenic determinant in the S protein that is deleted in PRCV have been developed to differentiate TGEV and PRCV (Jabrane *et al.,* 1992; Sestak *et al.,* 1999; López *et al.,* 2009). PEDV is not serologically related to TGEV or PRCV. Jung *et al. (*2003) recommended the multiplex RT-PCR as a monitoring and diagnostic tool to confirm the presence of PEDV and TGEV in formalin-fixed, paraffin-embedded tissues.

Apart from the epidemic acute outbreaks, where the TGEV prevalence and mortality is close to 100%, many studies focused on the prevalence of antibodies or fecal shedding of TGEV and PEDV worldwide (Table 7).

Rotavirus

Rotaviruses are RNA viruses of the *Reoviridae* family, which replicate mainly in the small intestine and spread mainly via the fecal-oral route. The rotaviral genome consists of 11 segments of double stranded RNA coding for six viral structural and six non-structural proteins (Matthijnssens *et al.,* 2008). The viral particles are composed of a capsid which contains three layers of viral proteins (VP) described as the outer (glycoprotein VP7 and non-glycosilated protease-sensitive VP4), intermediate (VP6), and inner (VP2) layers. VP1-3 are the core proteins (Yuan *et al*., 2006).

Rotavirus A is common in piggeries and is more prevalent in piglets from 1 to 3 weeks old and soon after weaning (Atii *et al.,* 1990). In pigs positive for rotavirus A, Halaihel *et al. (*2010) found that diarrhea is more likely to occur in the ones younger than 8 weeks old. The prevalence of infection and disease is favored by predisposing factors such as immunological quality and quantity of colostrum intake, nutrition and the immune status of the sows, poor sanitary conditions in pens and around the piggeries, and high population density (Steel and Torres-Medina, 1984; Atti *et al.,* 1990; Zijlstra *et al.,* 1999; Barreiros *et al.,* 2003). Additionally, the continuous exposure of pigs to rotaviruses is favored by their resistance in the environment as they maintain infective for 32 months at 10°C in stool specimens (Ramos *et al.,* 2000).

Porcine rotaviruses are antigenically diverse. The two outer capsid proteins, VP7 (G genotype) and VP4 (P genotype), independently elicit serotype-specific neutralizing immune responses that are believed to play an important role in protection against recurrent infections (Santos and Hoshino, 2005). Based on the differences in nucleic acid sequences of the outer capsid VP7 and VP4 encoding genes, 23 G and 31 P genotypes of rotavirus A have been described (Abe *et al.,* 2009; Ursu *et al.,* 2009). In pigs, 10 G types (1-6, 8-10 and 11) and 7 P types (5-8, 13, 9 and 23) of rotavirus A have been associated with diarrhea (Martella *et al.,* 2001; Barreiros *et al.,* 2003). Group A rotavirus infection has been recognized to occur in both enzootic and epizootic forms of swine diarrhea, resulting in serious economic losses in the suckling and weaning piglet population of commercial piggeries (Martella *et al.,* 2007; Kim *et al.,* 2010a).

The rotaviral replication in the villous epithelial cells cause malabsorption due to loss of absorptive cells and villous atrophy, which is the more accepted mechanism of rotavirus induced diarrhea in pigs (Greenberg and Estes, 2009). Rotavirus also induces an intestinal inflammatory response that may contribute to a secretory-type diarrhea (Zijlstra *et al.,* 1999), evokes intestinal fluid and electrolyte secretion by activation of the nervous system in the intestinal wall as evidenced by the use of four enteric nervous system inhibitor drugs (Lundgren *et al.,* 2000). The rotaviral non-structural protein 4

induces diarrhea in a similar way as *E. coli* STa by activating guanylate cyclase (Ball *et al.,* 1996; Kavanagh *et al.,* 2010).

Colostrum-deprived piglets inoculated with rotavirus 24 hours after birth develop profuse diarrhea with high mortality (63%). Interestingly, when these piglets were re-grouped with their colostrum-fed litter mates, the later got infected and developed diarrhea with a mortality of only 8% and decreased weight gains. Piglets recovered from rotavirosis can excrete the virus up to 21 days post-infection and the severity of the infection is age dependent, and piglets inoculated at 5-14 days old developed diarrhea but suffered a low mortality rate (Svensmark *et al.,* 1989a). The highest rotavirus prevalence occurs most frequently in litters from primiparous sows (Svensmark *et al.,* 1989b). Sows are easily infected with rotavirus by contact with an infected litter, but they do not show signs of diarrhea, meaning that they are an important source of infection to their offspring (Svensmark *et al.,* 1989a). Moreover this contact leads to a better transfer of anti-rotavirus immunoglobulins through colostrum in multiparous sows.

Field studies diverge concerning the pathogenicity of porcine rotaviruses. Rotavirus A was identified in feces of 43 out of 96 (44.8%) piglets suffering from acute gastroenteritis, while none of 41 non-diarrheic piglets were positive ($P<0.01$) in Nigeria (Atii *et al.,* 1990). Rotavirus A was the most prevalent enteropathogen associated with diarrhea in piglets from Japan (Katsuda *et al.,* 2006). In contrast, in Argentina, Parra *et al. (*2008) reported that only 5/30 1-45-day-old pigs positive to Rotavirus had diarrhea. These statements mean that in every geographic area or management system rotavirus can behave different, for example, in South Korea rotavirus A shedding in pig feces increases during the summer season (Kim *et al.,* 2010a).

The clinical signs of rotaviral enteritis are not pathognomonic. Worldwide, electron microscopy, immune electron microscopy, immunohistochemistry, immunofluorescence, ELISA, virus isolation, latex agglutination, dot blot hybridization, RNA electropherotyping, and RT-PCR have been employed to confirm rotaviral diagnosis (Yuan *et al.,* 2006). Electron microscopy is a highly specific method (Rodák *et al.,* 2009). ELISA is a reliable technique for the detection of rotaviral antigens in fecal samples or intestinal contents and is specially standardized and available for the diagnosis of porcine rotavirus A (Ingezim Rota DAS 1.1.RT.K.2 DAS-ELISA) with similar results as electron microscopy (Goyal *et al.,* 1987; Rodák *et al.,* 2004). Electrophoretyping of viral RNA isolated from feces is a useful technique, but it has to be confirmed by serological or nucleic acid-based methods (Mattion *et al.,* 1989; Rácz *et al.,* 2000). A variety of serological test have been performed and the ones that study the immunoglobulin isotypes are of help when detecting active or recent infections (Yuan and Saif, 2002). Multiplex RT-PCR offers a

more sensitive and reliable method for G and P genotyping of group A rotavirus (Martella *et al.,* 2001; Barreiros *et al.,* 2003). Chizhikov *et al. (*2002) recommended the oligonocleotide microarray hybridization for the identification of the G genotypes of all rotavirus strains combining RT-PCR and DNA-DNA hybridization. On swine stool specimens, Kang *et al. (*2007) suggested the use of immunochromathographic assays that can separately and accurately detect porcine rotaviruses, TGEV and PEDV.

In pigs, multiple rotavirus serogroups and serotypes have been detected worldwide (Table 8).

Table 7. Occurence of TGEV and PEDV detected by assays applied to serum and feces of pigs worldwide.

Groups & age	Tested	%		Assay	Country/Reference
		TGEV	PEDV		
Serum					
-	665	0	-	VN	U.S.A./Woods *et al.,* 1990
Sows	-	0.6	-	Competitive ELISA	Great Britain/Brown and Paton, 1991
-	229	89.9	-	ELISA	U.S.A./Phillips and Westerman, 1991
Breeding pigs	6000	1.27	-	ELISA	Spain/Cubero *et al.,* 1993
Pigs in positive farms	-	5-60	-	ELISA	Spain/Cubero *et al.,* 1993
Pigs in abattoirs	5.337	0	-	ELISA	South Africa/Williams *et al.,* 1994
>7 months feral swine	117	0	-	IFAT	U.S.A./Saliki *et al.,* 1998
Wild boars	134	1	-	IFAT	Czech Republic/Sedlak *et al.,* 2008
Wild boars	178	0	0	ELISA	Slovenia/Vengust *et al.,* 2006
-	263	12.5	-	ELISA	Japan/Miyazaki *et al.,* 2010
Diarrheic feces					
1-15 d.	749	59.2	-	FAT and EM	Canada/Morin *et al.,* 1983
0-21 d.	1258	0.9	50.4	RT-PCR	South Korea/Chae *et al.,* 2000
1-7 d.	33	0			
8-14 d.	50	4			
15-21 d.	19	10.5		EM	Germany/Wieler *et al.,* 2001
22-28 d.	16	0			
36-42[w] d.	31	51.6			
1-7 d.	100	6	-	FAT and IHC	U.S.A./Yaeger *et al.,* 2002
1-14 d.	157	2.5	13.5	RT-PCR	South Korea/Song *et al.,* 2006
1-21 d.	153	0	0	RT-PCR	Japan/Katsuda *et al.,* 2006
22-35[w] d.	116	0	0	RT-PCR	Japan/Katsuda *et al.,* 2006
2-28 d.	68	52	41.8	IC	Czech Republic/Czanderlova *et al.,* 2010

[w], weaned; d., days old.

Table 8. Worldwide occurrence (%) of Rotavirus in feces of diarrheic pigs during last decade.

Age group	Tested	%	A	B	C	Assay	Country/Reference
1-60 d. [DN]	165	35.3	100	NT	NT	PAGE, ELISA, RT-PCR	Brazil/Rácz *et al.,* 2000
1-7 d.	100	42	100	NT	NT	ELISA	U.S.A./Yaeger *et al.,* 2002
1-7 d.	33	0	NT	NT	NT	EM	Germany/Wieler *et al.,* 2001
8-14 d.	50	2					
15-21 d.	19	5.3					
22-28 d.	16	25					
36-42 d.	31	0					
< 7 d.	19	53	100	NT	NT	PAGE	Brazil/Barreiros *et al.,* 2003
8-21 d.	20	60					
> 21 d.	60	62					
1-7 d.	60	81.7	60-70*	NI	NI	RT-PCR	Japan/Katsuda *et al.,* 2006
8-14 d.	44	61.4					
15-21 d.	46	60.9					
22-28 d.	40	77.5					
29-35 d.	46	43.5					
1-14 d.	157	10.8	100	NT	NT	RT-PCR	South Korea/Song *et al.,* 2006
Piglets	175	22.3	100	NT	NT	ELISA in fecal samples	Thailand/Khamrin *et al.,* 2007
1-3 m.	102	-	71.5	NT	31.3	RT-PCR	Italy/Martella *et al.,* 2007
	86**		81.3		25.5	EM, RT-PCR	
< 45 d. [DN]	905	3.3	100	NT	NT	PAGE, ELISA, RT-PCR	Argentina/Parra *et al.,* 2008
< 3 w.	-	50	100	NT	NT	RT-PCR	Slovenia/Steyer *et al.,* 2008
3-10 w.		35.7					
> 10 w.		46.2					
Piglets	476	18.9	100	NT	NT	ELISA (fecal VP6)	Czech Republic/Rodák *et al.,* 2009
	21***	76.2	NT	14.3	76.2	PCR	
0-4 w. [DN]	45	26.7	100	NT	NT	RT-PCR	Spain/Halaihel *et al.,* 2010
4-8 w.	19	47					
8-16 w.	83	14.5					
16-24 w.	63	6.3					
3-70 d.	475	38.3	100	NT	NT	RT-PCR, nested PCR	South Korea/Kim *et al.,* 2010a
1-7 d.	30	13.3	NI	NI	NI	PAGE	Brazil/Cruz *et al.,* 2010
2-28 d.	98	30.6	100	NT	NT	IC	Czech Republic/Czanderlova *et al.,* 2010

A, B and C, groups of Rotavirus (%); [DN], diarrheic and non-diarrheic pigs.*, from the positives; **, positives by EM; ***, samples EM^{+}/ VP6 $ELISA^{-}$; NI, no information; NT, not tested; d., days old; w., weeks old, m., months old.

Cryptosporidium

The contribution of *Cryptosporidium* to piglet diarrhea should not be ruled out as experimental infections have revealed its potential pathogenicity (Suárez-Luengas *et al*., 2007). In two series of experimental infections to compare the infectivity of the *Cryptosporidium* pig genotype I (*C. suis*) and *C. parvum* in piglets, Enemark *et al. (*2003ab) reported that infection with *C. suis* caused no or very mild clinical signs whereas *C. parvum* provoked diarrhea. The species or genotypes of *Cryptosporidium* can be distinguished on the basis of the small subunit ribosomal RNA gene sequence (Xiao *et al.,* 1999).

It has been suggested that the withdrawal of protective maternal antibodies and the stress of weaning renders weaners particularly susceptible to infection (Hamnes *et al.,* 2007). As referred by Sanford (1987), Izumiyama *et al. (*2001) and Ryan *et al. (*2003), the higher positivity of pigs to *Cryptosporidium* occurs in the post-weaning period and Chen and Huang (2007) reported that the youngest pig excreting oocysts was 3 days old, and the oldest pig was 3.5 years old. Sows could maintain the parasite life cycle in piggeries, even in the absence of piglets, because stress associated with pregnancy and hormonal changes may increase susceptibility to infection (Zintl *et al.,* 2007). Recently Chen and Huang (2007) showed that *Cryptosporidium* isolated from pigs in eastern China belonged to the *C. parvum* 'mouse' genotype indicating that rodents as sows play a role in swine cryptosporidiosis epidemiology as reservoirs.

The specific mechanism by which *C. parvum* induces diarrhea has not been identified. Many molecules like CSL, a 1.300-kDa conserved apical complex glycoprotein, are crucial for the adherence of sporozoites and merozoites to the gut epithelium. *Cryptosporidium* gets internalized into the enterocytes and locates in a membrane-bound compartment on the apical surface. Therefore, direct damage of the brush border membrane, which provokes structural villous atrophy, and parasite derived molecules cause malabsorption and a sequence of biochemical/immunological changes like increase of secretogenic prostaglandins leading to diarrhea (Okhuysen and Chappell, 2002). Worldwide many studies have associated this *Apicomplexa* with swine diarrhea. Chen and Huang (2007) found in China a positive correlation between oocyst positivity and diarrhea, and *Cryptosporidium* prevalence was not significantly different between pre and post-weaned piglets. Hamnes *et al. (*2007) reported in Norway a significantly higher prevalence of diarrhea among the *Cryptosporidium* positive litters than among the negative ones. In Serbia it was reported that all *Cryptosporidium* positive nursing piglets had diarrhea, whereas in post-weaned piglets and adults *Cryptosporidium* infection was asymptomatic (Mišic *et al.,* 2003). In Mexico, Nevarez *et al. (*1997) implicated *Cryptosporidium* as infectious cause of enteritis in three 10-week-old weaned piglets from a crowded herd with bad sanitation and subjected to massive

treatment with several antibiotics. Conversely, in 20 out of 48 diarrheic pigs, *C. parvum* was not determined to be the only cause of diarrhea, suggesting that it may act in concert with other agents (i.e. *E. coli, I. suis, Salmonella*, adenovirus) to induce or exacerbate clinical disease and suggesting a strong possibility of subclinical infection (Sanford, 1987). Similarly, Quilez *et al. (*1996), Ryan *et al. (*2003), Guselle *et al. (*2003) and Vítovec *et al. (*2006) did not associate *Cryptosporidium* infection with swine diarrhea. Enemark *et al. (*2003a) found that a mixed infection of rotavirus and *Cryptosporidium* caused a dramatic aggravation of diarrhea. Vítovec *et al. (*2006), Maddox-Hyttel *et al. (*2006), and Johnson *et al. (*2008a) reported that *Cryptosporidium* occurs more frequently in weaned pigs than in sucking piglets.

Table 9 shows the fecal occurrence of *Cryptosporidium* in pigs raised worldwide during the last decade and several diagnostic techniques, which have been applied for its identification.

Isospora

The coccidian protozoa *Isospora* is one of the most common enteropathogens amongst suckling piglets in intensive pig production units, and is an important enteropathogen to be surveyed and controlled as the infection is associated with diarrhea (Harleman and Meyer, 1984; Vitovec and Koudela, 1990; Niestrath *et al*., 2002).

Piglets get infected by the ingestion of infectious oocysts from which the sporozoites emerge and invade the epithelial cells of the small intestine. Later, the course of the life cycle phases merogony (trophozoites, meronts, and merozoites), gametogony (macro and microgametocytes) and sporogony (immature oocyst) contribute to the destruction of enterocytes and to the colonization of the intestinal epithelium. Around four to five days after infection, piglets frequently have pasty to watery yellowish or grayish diarrhea for 3-7 days, dehydration, and weight loss (Mundt *et al.,* 2006). Clinical signs of isosporosis mostly appear in suckling piglets in the first three weeks of life with high morbidity and low mortality (Mundt *et al.,* 2003).

Lindsay *et al.* (1992) and Otten *et al*. (1996) reported that isosporosis can occur in any farm, independently of their size or management system. Niestrath *et al*. (2002) reported that the rearing conditions and hygienic status on swine farms influence the prevalence of coccidian infections in piglets.

Traditionally, sows have been thought to be the main source of *Isospora* in a farm, but Karamon *et al.* (2007) in Poland demonstrated no correlation between the excretion of oocysts by the sows and the infection of piglets born from them. The farrowing pens contaminated with oocysts excreted by previous litters is considered to be the main source of infection, showing the importance of good sanitation (oocysts are resistant to most of disinfectants) and the use of self-cleaning floors in farrowing

facilities for isosporosis prevention (Lindsay *et al*., 1989; Niestrath *et al*., 2002; Karamon *et al*., 2007). In that context, Niestrath *et al*. (2002) commonly found *I. suis* in farms which employed straw bedding.

The general laboratory methods which included staining and or visualization of oocyst morphology by light microscopy have been employed to identify *Isospora* but their sensitivity is negatively influenced by the high fat content of piglets feces and because oocysts are not always excreted or are excreted intermittently. Daugschies *et al*., 2001 reported that autofluorescence microscopy is more sensitive than bright field microscopy for the detection of *I. suis* oocysts after flotation or in direct smears. Recently a PCR–RFLP assay based on the rDNA ITS-1 region has been developed to identify *Isospora* (Samarasinghe *et al*., 2007; Johnson *et al.*, 2008b). Table 10 shows the assays mostly employed for the identification of porcine *Eimeriidae* and their prevalence worldwide.

Table 9. Worldwide reported fecal occurrence (%) of *Cryptosporidium* in pigs during last decade.

Groups	Tested	%	*C. suis*	*C.* Gen II	*C. parvum*	*C. muris*	Assay	Country/Reference
1m.	213	32.3			x		Formol-ether concentration	Japan/Izumiyama *et al.,* 2001
3 m.	19	42.1			x		Immunofluorescent staining (A)	
6 m.	252	0.4			x			
Suckers and weaners	287	1.4			x		Carbolfuchsin staining	Germany/Wieler *et al.,* 2001
Adults	589	10.5			x		Acid-fast staining	South Korea/Yu and Seo, 2004
< 5 w.	3368	5.7	x				Aniline-carbol-methyl violet staining	Czech Republic/Vítovec *et al.,* 2006
> 6 w.	835	24.1						
Sows	135	0					Sheather's flotation, PCR	
< 1 m.	488	6					Immunofluorescent staining	Denmark/Maddox-Hyttel *et al.,* 2006
1-4 w.	504	71						
Sows	245	4						
1-7 d.	60	0					Flotation[D]	Japan/Katsuda *et al.,* 2006
8-14 d.	44	0						
15-21 d.	46	0						
22-28 d.	40	2.5						
29-35 d.	46	28.3						
4-10 w.	127	15	x	x			Auramine phenol staining	Ireland/Zintl *et al.,* 2007
10-24 w.	121	7.4	x				Sheather sugar flotation, PCR	
6-12 m. gilts	15	6.7						
4 y. sows	75	13.3	x	x	x	x		
3-4 y. boars	4	0						
1-2[W] m.	75	30.7	x	x			Formol-ether concentration, PCR	Spain/Suárez-Luengas *et al.,* 2007
2-6 m.	42	11.9		x				
Sows	25	16	x					
Suckers	183	10.4			x		Acid-fast staining, PCR	China/Chen and Huang, 2007
Weaners	317	12.9						
Fatteners	536	13.8						
Boars	91	8.8						
11d. - 3 w.	123	10.6	x				PCR	Australia/Johnson *et al.,* 2008a
4 w. - 6 m.	156	32.7	x	x				
Sows	10	0						

Table 9 (continuation). Worldwide reported fecal occurrence (%) of *Cryptosporidium* in pigs during last decade.

Groups	Tested	%	*C. suis*	*C.* Gen II	*C. parvum*	*C. muris*	Assay	Country/Reference
Suckers	116	21.8	x				Aniline-carbol-methyl violet staining, PCR	Czech Republic/Kvác *et al.,* 2009
Weaners	131	29	x	x		x		
Fatteners	123	17	x	x		x		
Sows	40	2.5						
<6 m.	105	11.4					Formol-ether concentration and acid-fast staining	Turkey/Uysal *et al.,* 2009
>6 m.	133	6.7						
0-3 w.	20	20	x*	x*	x*		Immunofluorescent staining, PCR	UK/Featherstone *et al.,* 2010**
4-7 w.	36	38.9						
8-13 w.	93	48.4						
14-24 w.	119	40						
>24 w.	39	30.8						

[w,] weaned pigs; d., days old; w., weeks old; m., months old; y., years old; *, information from the overall sample; [D], all piglets had diarrhea; (A), all pigs were asymptomatic; **, *Cryptosporidium* pig genotype II was detected in 25 out of 39 (64.1%) sequenced isolates. *C. parvum* was identified in eight (20.5%) isolates, and *C. suis* was identified in six (15.4%) isolates.

Table 10. Worldwide reported fecal occurrence (%) of *Eimeriidae* in pigs during last decade.

Group & age	Tested	*I. suis*	*Eimeria* spp.	*Eimeriidae*	Assay	Country/Reference
1-7 d.	41	7.3	NI		Merthiolate iodine formalin concentration	Germany/Wieler *et al.,* 2001
8-14 d.	102	39.2	NI			
15-21 d.	40	32.5	NI			
22-28 d.	18	11.1	NI			
29-35 d.	4	25	NI			
36-42 d.	82	1.2	NI			
10-20 d.	300[LI]	33	NI		Culture on 2.5% potassium dichromate, Telemann sedimentation method	Belgium/Leten *et al.,* 2002
Diarrheic	264[LI]	49.2	NI		Sedimentation-Flotation, McMaster	Germany/Niestrath *et al.,* 2002
Non-diarrheic	81[LI]	22	NI			
4-10 w.	265[NT]			5.4	NaCl Flotation	China/Weng *et al.,* 2005
11-17 w.	259			18.9		
18-23 w.	231			20.3		
Sows	268			51.1		
Boars	85			61.1		
2-30 w.	133[N]	76.7	NI		NaCl/sugar Flotation	Germany, Austria, Switzerland /Mundt *et al.,* 2005
1-7 d.	60[D]			0	Flotation	Japan/Katsuda *et al.,* 2006
8-14 d.	44[D]			36.4		
15-21 d.	46[D]			26.1		
22-28 d.	40[D]			10		
29-35 d.	46[D]			26.1		
1-7 w.	514	10	7		Sodium acetate-acetic acid-formalin (SAF) concentration.	Germany/Damriyasa and Bauer, 2006
5-28 d.	571[L, LI]	31.7	1.4		NaCl/sugar Flotation, McMaster, culture on 2.5% potassium dichromate	Poland/Karamon *et al.,* 2007
5-28 d.	171[M, LI]	18.1	0.6			
5-28 d.	38[S, LI]	13.2	28.9			
Sows	159[L]	8.2	4.4			
Sows	98[M]	5.1	5.1			
Sows	10[S]	0	40			
10 d. - 3 w.	123	6.4	NI		PCR- RFLP	Australia/Johnson *et al.,* 2008b
4 w. - 6 m.	156	16.3	NI			
Sows	10	0	NI			

Table 10 (continuation). Worldwide reported fecal occurrence (%) of *Eimeriidae* in pigs during last decade.

Group & age	Tested	*I. suis*	*Eimeria* spp.	*Eimeriidae*	Assay	Country/Reference
7-15 d.	-	32.8	NI		Sheather's sugar Flotation, Giemsa and Ziehl-Nielsen staining	Servia/Savic *et al.,* 2010
d. 13	-	44.3	NI			
Diarrheic	-	37.4	NI			
Non-diarrheic	-	15	NI			
10-18 d. [LI]	580	37	NI		NaCl/glucose Flotation	Belgium/Laitat *et al.,* 2010
Suckers	118	65.6[D]	NI		NaCl/glucose Flotation, McMaster	Greece/Skampardonis *et al.,* 2010
1-7 d.	30[D]	0	NI		Flotation	Brazil/Cruz *et al.,* 2010
Suckers	709[LI]	26.4	NI		Cornell-Wisconsin flotation	Canada/ Aliaga-Leyton *et al*., 2011

[L, M and S], from large (>100 sows), medium (25-100 sows) or small (<25 sows) farms; [LI], litters; [D], piglets were tested during all suckling period; [N], never treated herds; [D], all tested piglets had diarrhea; [NT]; no regular regime of anti-parasite treatment.
Studies reported as *Eimeriidae* did not make the differential identification of *Eimeria* spp. and *I. suis* oocysts.

Aims of the study

Aims of the study

Porcine pre- and post-weaning diarrhea is a multi-factorial disease responsible for tremendous economic losses to the pig industry worldwide, and several infectious agents play a major role in the pathogenesis (Wieler *et al*., 2001; Katsuda *et al*., 2006).

In Cuba, swine veterinary practitioners have to face a high occurrence and a yearly increased incidence of porcine diarrhea, as well as limitations in the specific identification of its etiology, leading to uncontrolled administration of antibiotics, insufficient prevention programs, and scarce information on its epidemiology. In 2008, the Cuban Institute for Swine Research reported gastroenteric diseases (an unspecific terminology) to be the cause of 31% and 37% of the total piglet's mortality during the pre- and post-weaning periods, respectively (Cabrera and García, 2009).

Therefore, in order to contribute to the epidemiological characterization of enteropathogens of young pigs in Cuba, this PhD thesis focused on

- (Chapter 2) the identification of enteropathogens currently associated with pre- and post-weaning diarrhea,
- (Chapter 3) the antibiotic resistance and genetic relatedness of pathogenic *E. coli*,
- (Chapter 4) the seroprevalence of specific antibodies against *E. coli* fimbriae and
- (Chapter 5) the genetic susceptibility of the commercial breed currently raised in Cuban piggeries to enteric colonization by fimbriated *E. coli*.

With all these data and results we want to propose a strategy to remediate the problem of pre- and post-weaning diarrhea in Cuba.

Chapter 2

Association of toxigenic E. coli *and several other pathogens with porcine pre- and post-weaning diarrhea in Villa Clara province, Cuba.*

Based on:

de la Fé Rodríguez PY, Maroto Martin LO, Cruz Muñoz E, Butaye P, Goddeeris BM, Cox E, 2011. Several enteropathogens are associated with porcine pre-weaning and post-weaning diarrhea in Villa Clara province, Cuba. Submitted to Tropical Animal Health and Production (manuscript number TROP-2977).

2.1 Abstract

Intestinal contents of suckling (n = 45) and recently weaned (n = 45) diarrheic pigs from Villa Clara province, Cuba, were tested for the presence of important viral, bacterial, and parasitic enteropathogens. At least one enteropathogen was detected in 64.4% and in 42.2% of suckling and weaned pigs, respectively. ETEC was significantly the most frequent pathogen in suckling piglets (28.9%; $p<0.05$) as well as in weaned pigs (22.2%), and most of isolates were either STa^+/STb^+ or $F4^+/STa^+/STb^+$. The overall occurrence of the rest of pathogens was 10% for TGEV and *C. parvum*, 6.7% for rotavirus A and *I. suis*, 5.6% for α-toxigenic *C. perfringens*, 3.3% for VTEC, and 2.2% for *Salmonella enterica* subspecies *enterica* serotype Newport. TGEV and α-toxigenic *C. perfringens* were only identified in suckling piglets, while *S.* Newport and VTEC were only detected in weaned pigs. PEDV, β-toxigenic *C. perfringens, Eimeria* spp., and helminths were not identified during this study. Twelve out of 48 enteropathogen positive pigs (25%) carried mixed infections. ETEC was present in 10 out of 12 mixed infections, and TGEV infections were never combined. The results obtained herein demonstrate that several enteropathogens, either alone or as part of a combined infection, are associated with porcine pre- and post-weaning diarrhea in Villa Clara province, Cuba. It is necessary to implement actions aimed on the surveillance, prevention, and control of swine enteropathogens in Cuban piggeries.

2.2 Introduction

Many infectious agents, alone or in combination, can play an important role in the pathogenesis of porcine pre- or post-weaning diarrhea, which are multi-factorial diseases negatively affecting performance of young pigs and swine production efficiency (Katsuda *et al.,* 2006). Rotaviruses, TGEV, PEDV, ETEC, toxigenic *C. perfringens*, and *Coccidia* have been identified as the most associated pathogens with piglet's diarrhea worldwide (Chae *et al.,* 2000; Wieler *et al.,* 2001; Barrera *et al.,* 2005; Straw *et al.,* 2006; Katsuda *et al.,* 2006).

In 2008, the Cuban Institute for Swine Research reported that gastroenteric diseases caused 31% and 37% of the total piglet's mortality during the pre- and post-weaning periods, respectively (Cabrera and García, 2009). However, there is scarce information on the epidemiology of porcine enteropathogens because in most of the diarrhea outbreaks, diagnosis is restricted to clinical and macro-pathological examinations (Cabrera and García, 2009). Also, limited research with a focus on porcine enteropathogens has been performed during the last 20 years in Cuban piggeries (Fuentes *et al.,* 2001; Barrera *et al.,* 2005; Blanco *et al.,* 2006).

Therefore, in order to contribute to the epidemiological characterization of porcine infectious diarrhea in Cuba, the present study undertook the differential identification of enteropathogens that could be associated with porcine pre- or post-weaning diarrhea in the Villa Clara province.

2.3 Materials and methods

Survey design

Along the period from May to June 2008, the department of Veterinary services of the Company of Pork Production (GRUPOR) of Villa Clara, which is the province that produces most pork in Cuba (González *et al*., 2007), assigned 90 diarrheic piglets for being surveyed for enteropathogens. Piglets were distributed in 6 of the 8 largest indoor piggeries free of hog cholera outbreaks in the province, otherwise access and sampling is forbidden due to biosecurity rules. The herd of the 6 piggeries together comprised 5252 sows and 7455 suckling piglets; the number of newly weaned piglets was not controlled. All piggeries followed a similar continuous flow management (farrow-to-finish) without straw bedding, they had similar facilities, they used the same antibiotics, and they had shared breeding stock animals routinely. Pregnant sows were housed individually (Fig. 1), and at the end of gestation they were moved up to elevated farrowing crates with completely perforated plastic flooring (Fig. 2 and 3). After weaning, piglets were housed in groups of 10 to 20 in elevated pens with perforated floors (Fig. 4).

On the appointed day to sample in every piggery, the responsible veterinarian was asked to select during the early morning inspection 6-9 litters (2 to 25-day-old) and 6-9 newly weaned pens

(26 to 48-day-old) showing signs of watery diarrhea to complete 15 sampling places spread over the farrowing and weaning houses. Then, one piglet per litter or per pen was randomly catched, and selected and euthanatized if sign of diarrhea was present in the perineum. Before euthanasia, blood was collected and serum was obtained as described in Chapter 4. Piglets and sows were not under antibiotic therapy or vaccinated against enteropathogens.

Figure 1. Individual housing of pregnant sows in a Cuban piggery.

Figure 2. Elevated farrowing crates with completely perforated plastic flooring currently used in Cuban piggeries.

Figure 3. Inside and outside of a farrowing installation in a Cuban piggery.

Figure 4. Weaned pigs raised in elevated pens with perforated floors in a Cuban piggery.

Specimens (i.e. intestinal contents, serum, blood) were maintained in an insulated transport container filled with refrigerant gels until arrival to the laboratory, and Stuart Transport Medium modified by Ringertz was employed for keeping bacteria alive. For the identification of pathogens for which commercial diagnostic kits were planned to be used, intestinal contents and sera were

aliquoted and stored at -20°C until the kit application to all samples. Intestinal contents started to be processed quickly for bacterial culture and general parasitological methods.

Bacteriological identification

In order to isolate *E. coli*, intestinal contents were inoculated on McConkey agar for 18 h at 37°C in aerobic atmosphere. Thereafter 3-5 lactose-fermenting colonies per sample were separately re-inoculated on McConkey agar. Then, for biochemical identification one colony per plate was inoculated on Luria agar for 18 h at 37°C, and was later identified as *E. coli* if it was able to ferment lactose and glucose with gas production (tested on Kligler Iron agar), to produce indole from tryptophan [visualized employing the Kovac's reagent: 50 g/l of 4-(dimethylamino)benzaldehyde + 75% of 11 M 1-butanol + 25% of concentrated 12 M HCl], and also if did not hydrolyze aesculin (tested on Bile Aesculin agar; Moyaert *et al*., 2006; Bruggeman *et al*., 2008) and if did not utilize citrate (tested on Simmons citrate agar). Intestinal contents were also plated onto 5% sheep blood agar and a maximum number of 3 hemolytic colonies were further processed as described above. Finally, one hemolytic *E. coli* isolate per pig was selected for being tested for fimbriae (F4, F5, F6, F18, and F41) and toxins (STa, STb, LT, and STx2e) encoding genes.

Identification of genes encoding virulence factors in E. coli *by multiplex-PCR*

From an overnight culture on Brain Heart Infusion agar at 37°C, 3-4 colonies were suspended in 100 µl sterile distilled water, and heated for 15 min at 90°C in warm water bath. Next, the bacterial lysate was chilled on ice for 5 min and centrifuged at 14,000×*g* for 1 min. Aliquots of the supernatant were stored at -20°C and used as template DNA. The reaction mixture (20 µl) was composed by 2 µl of template DNA, 0.5 µM of each primer (Table 1; Bruggeman *et al.,* 2008), 0.2 mM of each dNTP (Pharmacia), 2.5 U of Taq polymerase (Ampli Taq Gold, Perkin Elmer), 1X of Ampli Taq Gold buffer (10 mM Tris pH 8.3, 5 mM KCl), and 5 mM of $MgCl_2$ (Perkin Elmer). The PCR was made on a termocycler Gene Amp PCR System 9600 (Perkin Elmer) by pre-denaturation of the mixture at 90°C for 3 min, followed by 30 cycles of amplification including denaturation at 90°C for 1 min, annealing at 55°C for 1 min plus increment of 3 sec/cycle, and primer extension at 70°C for 2 min; the final extension was at 70°C for 10 min. The employed positive controls were *E. coli* B41 ($F5^+/F41^+/STa^+$), E68 ($F4^+/LT^+/STb^+$), and 107/86 ($F18ab^+/STx2e^+$). *E. coli* HB101 was the negative control. All PCR products were run at 120 V and 190 mA for 90 min in 3% Nusieve 3.1 agarose gel in 0.5X TBE buffer [10X TBE stock solution: 108 g Tris (Merck) + 55 g Boric Acid (Merck) + 9.3 g EDTA (Merck) + 1000 ml distilled water]. Staining was made with ethidium bromide (Sigma).

Table 1. Primer sequences used in the multiplex-PCR for detecting genes encoding virulence factors in *E. coli*.

Target gene	Virulence factor	Oligonucleotide sequence of forward and reverse primers (3'→5')	Amplicon size (bp)
estII	STb	TGCCTATGCATCTACACAAT CTCCAGCAGTACCATCTCTA	113
estIa	STa	CAACTGAATCACTTGACTCTT TTAATAACATCCAGCACAGG	158
fanA	F5	AATACTTGTTCAGGGAGAAA AACTTTGTGGTTAACTTCCT	230
eltB	LT	GGCGTTACTATCCTCTCTAT TGGTCTCGGTCAGATATGT	272
fedA	F18	TGGTAACGTATCAGCAACTA ACTTACAGTGCTATTCGACG	313
fasA	F6	GTAACTCCACCGTTTGTATC AAGTTACTGCCAGTCTATGC	409
faeG	F4	GAATCTGTCCGAGAATATCA GTTGGTACAGGTCTTAATGG	499
fimF41a	F41	AGTATCTGGTTCAGTGATGG CCACTATAAGAGGTTGAAGC	612
Stx2e	STx2e	AATAGTATACGGACAGCGAT TCTGACATTCTGGTTGACTC	733

For identification of *Salmonella* spp., intestinal contents were inoculated on brilliant green agar (BGA) and in 5 ml of Rappaport-Vassiliadis broth for 18 h at 37°C in aerobic atmosphere, and then a re-inoculation on BGA was performed. Non-lactose-fermenting colonies from BGA were further biochemically identified and re-plated on brain heart infusion agar. Thereafter a slide agglutination was carried out using polyvalent (A+B+C1+C2+D+E1+E2+E4+F groups) and monovalent (A, B, C1, and C2 groups) *Salmonella* antisera (Company of biological products C.J. Finlay, Havana, Cuba). Positive isolates on slide agglutination were tested by the multiplex *Salmonella* DNA typing Premi®Test (Veterinary and Agrochemical Research Centre, Brussels, Belgium; Wattiau *et al.,* 2008).

The semi-quantitative detection of *C. perfringens* as well as the identification of the α- and β-toxins was performed on intestinal contents by a DAS-ELISA kit (BIO K 095, Bio-X Diagnostics Inc., Jemelle, Belgium). A sample was considered positive if it was positive for the bacterium and for at least one toxin.

Virus identification

The specific detection of TGEV and PEDV in intestinal contents was carried out by a qualitative chromatographic immunoassay kit (Anigen Rapid TGE/PED Ag test kit, Animal Genetics Inc., Suwon, South Korea). Complementary, sera of suckling piglets were tested for antibodies against TGEV and PRCV by a differentiating blocking ELISA kit (SVANOVIR® TGEV/PRCV-Ab, Svanova Biotech AB, Uppsala, Sweden), which allows the detection of three serological statuses: seronegative piglets for both coronaviruses, seropositive piglets for PRCV and seronegative for TGEV, and seropositive

piglets for TGEV. Rotavirus A was tested in intestinal contents by a DAS-ELISA kit (1.1.RT.K2, Ingenasa, Madrid, Spain).

Parasitological identification

Intestinal contents were examined for *Eimeriidae* and helminths by light microscopy using the direct smear and Sheather's sugar flotation methods. As previously described by Karamon *et al.* (2007), oocysts sporulation was induced in *Eimeriidae* positive samples to allow the differential identification of *Eimeria* spp. and *I. suis*. *C. parvum* antigen detection was carried out using a DAS-ELISA kit (BIO K 070, Bio-X Diagnostics Inc., Jemelle, Belgium).

Statistical analysis

The proportions of piglets infected by every enteropathogen in every age group (suckling piglets, newly weaned piglets, 2 to 13, 14 to 25, 26 to 36, and 37 to 48-day-old piglets) as well as in the overall sample were compared by the chi-square test in StatGraphics Plus Version 5.0. Differences were considered significant at $p<0.05$.

2.4 Results

Identified enteropathogens

At least one enteropathogen was identified in 29 out of 45 (64.4%) suckling piglets, and in 19 out of 45 (42.2%) weaned pigs (Table 2). PEDV, β-toxigenic *C. perfringens, Eimeria* spp., and helminths were not detected during this survey. The color of scours varied from milky to dark yellowish, and bloody diarrhea was not observed. Pathogenic *E. coli* (ETEC and VTEC) were the most frequent enteropathogens (Table 2), being isolated from intestinal contents of 28.9% of all pigs (n=90). The combination of genes encoding STa and STb was commonly detected as most of the pathogenic *E. coli* were either STa^+/STb^+ (31%) or $F4^+/STa^+/STb^+$ (19%; Table 3). One STa^+/STb^+ and all $F18^+$ or $F4^+/STa^+/STb^+$ isolates were beta-hemolytic.

The only isolated *Salmonella* spp. was *Salmonella enterica* subspecies *enterica* serotype Newport (serogroup C2) in 2 (4.4%) weaned pigs (Table 2), and in one of both cases the infection was combined with ETEC and *C. parvum* (Table 4).

The bacterium *C. perfringens* was detected in 66.7% (30/45) of suckling piglets, but only 16.7% (5/30) of these *Clostridium*-positive piglets were positive for α-toxin. It was noted that the α-toxin-positive intestinal contents contained a higher bacterium concentration (average OD_{450nm} = 1.679) than the toxin-negative ones (average OD_{450nm} = 0.990). In weaned piglets, *C. perfringens* was identified in 26.7% of the pigs, whereas toxins were not detected in this group.

Table 2. Percentage of diarrheic piglets infected by enteropathogens and enteric mixed infections per age group between May and June 2008 in the Villa Clara province, Cuba.

Age groups		No. of pigs	Enteropathogens																Enteric mixed infections		Total[a]	
			Rotavirus A		TGEV		ETEC		VTEC		*S.* Newport		α-toxigenic *C. perfringens*		*I. suis*		*C. parvum*					
			n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
Suckling	2-13-day-old	31	2	6.5	9	29*	7	22.6*	0	0	0	0	3	9.7	2	6.5	1	3.2	4	12.9	20	64.5
	14-25-day-old	14	2	14.3	0	0	6	42.9*	0	0	0	0	2	14.3	2	14.3	2	14.3	3	21.4	9	64.2
	Subtotal	45	4	8.9	9	20	13	28.9*	0	0	0	0	5	11.1	4	8.9	3	6.7	7	15.6	29	64.4
Weaned	26-36-day-old	24	1	4.2	0	0	5	20.8*	3	12.5	2	8.3	0	0	1	4.2	2	8.3	3	12.5	10	41.7
	37-48-day-old	21	1	4.8	0	0	5	23.8*	0	0	0	0	0	0	1	4.8	4	19	2	9.5	9	42.9
	Subtotal	45	2	4.4	0	0	10	22.2*	3	6.7	2	4.4	0	0	2	4.4	6	13.3	5	11.1	19	42.2
Total		90	6	6.7	9	10	23	25.6*	3	3.3	2	2.2	5	5.6	6	6.7	9	10	12	13.3	48	53.3

*, Enteropathogens frequency which is significantly higher ($p<0.05$) than the frequency of other enteropathogens for pigs of the same age group.
[a], Total of enteropathogen positive pigs per age group, pigs carrying mixed infections are counted as one.

Table 3. Association and diversity of fimbrial and toxin encoding genes among 26 pathogenic *E. coli* isolated from intestinal contents of suckling (s) and weaned (w) pigs with diarrhea in the Villa Clara province, Cuba.

Toxins / Fimbriae	**STa**		**STb**		**STa/STb**		**STb/LT**		**STx2e**		**None**		Total (%)
	s	w	s	w	S	w	s	w	s	w	s	w	
F4	-	-	-	-	3	2	-	-	-	-	-	1	6 (23)
F6	-	1	-	-	-	-	-	-	-	-	-	-	1 (4)
F18	-	-	-	-	-	-	-	2	-	3	-	-	5 (19)
F5/F41	1	-	-	-	-	-	-	-	-	-	-	-	1 (4)
None	1	-	1	2	6	2	1	-	-	-	-	-	13 (50)
Total (%)	3 (12)		3 (12)		13 (50)		3 (12)		3 (12)		1 (4)		26 (100)

Rotavirus A was detected in every age group, with a slightly higher occurrence (14.3%) in 14- to 25-day-old piglets (Table 2). TGEV was identified in 29% of 2- to 13-day-old piglets only. Most of suckling piglets (77.7%) were seropositive for TGEV, which was excreted by 2 out of 5 (40%) TGEV seronegative piglets, as well as by 5 out of 35 (14%) TGEV seropositive ones. Only one piglet was seropositive for PRCV and seronegative for TGEV, and 3 were seronegative for both coronaviruses.

Comparing different age groups, it seemed as if the 14- to 25-day-old piglets were more susceptible to enteropathogens than the others; this age group showed the highest occurrence of rotavirus A, ETEC, α-toxigenic *C. perfringens*, and *I. suis,* as well as the second highest of *C. parvum*. Also mixed infections were most frequent in this age group. However, it should be noted that the number of tested and therefore also the number of positive animals in this age group was low. Furthermore, the percentage of piglets showing enteric infections was as high in the 2-13-day-old age group.

Table 4. Mixed infections by enteropathogens identified in intestinal contents of suckling and weaned pigs suffering from diarrhea in Villa Clara province, Cuba.

Pigs	**Age (days)**	**Mixed infections**
Suckling	3	*C. parvum+* α-toxigenic *C. perfringens*
	5	rotavirus A + *E. coli* STb^+
	11	*I. suis* + α-toxigenic *C. perfringens*
	13	rotavirus A + *E. coli* STa^+/STb^+
	14	*C. parvum* + *I. suis* + *E. coli* $F4^+/STa^+/STb^+$
	15	*I. suis* + rotavirus A + *E. coli* $F4^+/STa^+/STb^+$
	16	*C. parvum* + *E. coli* Sta^+/STb^+
Weaned	34	rotavirus A + *E. coli* $F4^+/STa^+/STb^+$
	34	*I. suis* + *E. coli* $F4^+/STa^+/STb^+$
	36	*C. parvum* + *S.* Newport + *E. coli* STb^+
	41	*I. suis* + *E. coli* STa^+/STb^+
	46	*C. parvum* + *E. coli* $F4^+$

Infections by multiple enteropathogens were detected in 25% of enteropathogen positive pigs. Mixed infections were slightly more common in suckling piglets (7/12) than in weaned pigs (5/12; Table 4). *I. suis* infections were almost always (5/6) combined with other pathogens; similar results were observed for rotavirus A (4/6) and *C. parvum* (5/9). Alpha-toxigenic *C. perfringens* infections were only mixed with *C. parvum* or *I. suis*. TGEV infections were not mixed, while ETEC was present in 10 out of 12 combined infections.

2.5 Discussion

In Cuba, diarrhea markedly influences piglet's mortality (Cabrera and García, 2009). However, knowledge of which enteropathogens could be associated with this syndrome has been limited during the last 20 years. Therefore, the study reported herein updates on the frequency of enteropathogens associated with porcine pre- and post-weaning diarrhea in Cuban piggeries.

Several enteropathogens, either alone or as part of a mixed infection, were identified in 64.4% of suckling piglets and in 42.2% of weaned pigs with diarrhea. ETEC were more frequent ($p<0.05$) than other enteropathogens in all age groups, except in the 2- to 13-day-old piglets, in which they were as frequent as TGEV. Surveys aimed on the differential identification of enteropathogens in diarrheic piglets reported *I. suis* in Germany (Wieler *et al.,* 2001) and rotavirus in Japan (Katsuda *et al.,* 2006) as the most prevalent agents, the authors attributed a low occurrence of ETEC to vaccination programs and to the use of effective antibiotics. In Cuban piggeries, housing and management conditions are different, vaccination against ETEC has not been applied during the last years (Wong *et al.,* 1995), and there is a high resistance rate of porcine pathogenic *E. coli* to the routinely administered antibiotics (de la Fé Rodríguez *et al.,* 2012).

F4, F5, and F41 encoding genes have not been previously identified in *E. coli* isolates from diarrheic piglets in Cuba (Blanco *et al*., 2006). Furthermore, our study is the first to demonstrate the enterotoxin encoding genes (STa or STb) in $F4^+$ and $F41^+$ ETEC isolates from Cuba as their identification was not performed by Fuentes *et al.* (2001) and Lazo *et al.* (2005). LT was only identified in 3 isolates in the present survey; nevertheless, most of ETEC carried genes encoding ST enterotoxins, which are sufficient for causing diarrhea in neonatal (STa) and newly weaned (STb) pigs (Berberov *et al.,* 2004).

$F4^+/STa^+/STb^+$ ETEC as identified in our study, were also isolated from diarrheic piglets in South Korea (Chae *et al.,* 1998; Kwon *et al.,* 2002); in contrast, a strong association between F4 and LT encoding genes has been shown in ETEC associated with swine diarrhea worldwide (Frydendahl, 2002; Zhang *et al.,* 2007). The two $F18^+/LT^+/STb^+$ ETEC isolated from weaned pigs constitute the first report of $F18^+$ ETEC carrying LT encoding genes in Cuba, corresponding with previous findings in USA (Zhang *et al.,* 2007), and differ from the $F18^+/STx2e^+/STa^+/STb^+$ strains previously isolated in Villa

Clara by Blanco *et al.* (2006). Recently, a high seroprevalence of specific antibodies against F4 and F18 fimbriae was demonstrated in Cuban swine (de la Fé Rodríguez *et al.,* 2011), supporting the importance of $F4^+$ or $F18^+$ *E coli* in the pathogenesis of swine diarrhea or edema disease over the whole country. ETEC harboring the F6 fimbrial gene are often associated with pre-weaning diarrhea (Blanco *et al.,* 2006), but in the present survey F6 was only linked with post-weaning diarrhea in a low rate like reported by Frydendahl (2002).

Salmonella spp. were not as frequently isolated as ETEC; only 2 weaned pigs were infected by *S*. Newport. There is no previous information on the presence of this pathogen in Cuban piggeries. Fever, diarrhea, dehydration, and enteritis have been observed in calves, cows, and horses from which *S.* Newport was isolated (Poppe *et al.,* 2006), but more research on its role in the pathogenesis of piglet's diarrhea is needed (Zhao *et al.,* 2003). Katsuda *et al*. (2006), and other authors (see Tables 3 and 4, Chapter 1), could not isolate *Salmonella* in suckling and newly weaned pigs with diarrhea. Nevertheless, the *Enterobacteriaceae Salmonella* cause diarrhea and enterocolitis in pigs mostly from post-weaning to around 4 months of age, and *Salmonella enterica* subspecies *enterica* serotype Cholerasuis and serotype Typhimurium are the most commonly associated (Griffith *et al*., 2006). Recently Hur *et al*. (2011) reported that 42 *S.* Typhimurium isolated from diarrheic pigs aged from 2 to 72 days in South Korea carried diverse genes encoding antibiotic resistance and virulence factors. Therefore, it has been recommended that swine production systems have to be routinely surveilled for *Salmonella,* a zoonotic foodborne pathogen (Chiu *et al*., 2004).

In Cuba, the identification of *C. perfringens* toxins is not performed during the routine analysis of swine diarrhea. The higher concentration of this bacterium detected in α-toxin-positive intestinal contents when comparing with the α-toxin-negative ones, suggests the presence of active infectious processes due to α-toxigenic *C. perfringens* in these suckling piglets. Das *et al.* (2009) found a significant association of *C*. *perfringens* A with swine diarrhea in India. Here α-toxigenic *C. perfringens* was the single enteropathogen in 3 out of 5 positive piglets, whereas coinfection with *C. parvum* or *I. suis* was seen in both other piglets; Songer and Uzal (2005) stated that preceding lesions provoked by other enteropathogens can enhance enteric colonization by *Clostridium*. Beta-toxigenic *C*. *perfringens* was not identified, supporting the absence of reports on necrotic enteritis in the sampled piggeries which might be due to a β-toxin effect.

TGEV always occurred as a single infection of suckling piglets, and the associated diarrhea was not profuse and epidemic like in the classical TGE acute form. A milder form of TGE can be commonly observed (Morin *et al.,* 1983), and its clinical differentiation from coccidial, rotaviral, and ETEC infections is difficult, as clinical signs are less severe than those observed in the classic form. From 2003 on, epidemic TGE outbreaks have been reported in Cuba (Barrera *et al.,* 2005), but the milder behavior found in the present study, and the high amount of seropositive suckling piglets, indicates a

possible change to the endemic form and a high prevalence at herd level as the serological status of newborn piglets is influenced by maternal antibodies. Additionally, antibodies against PRCV were for the first time reported for Cuba during the present survey. So presence of these antibodies could also explain milder TGEV infections since it is well-known that an infection with PRCV induces antibodies able to neutralize and decrease the excretion of TGEV, inhibiting the presentation of epidemic TGE in PRCV positive herds (Cox *et al.,* 1993).

Four out of 6 rotavirus A infections were mixed with ETEC; interestingly, three of them occurred in suckling piglets. Rotaviruses are of importance in the pathogenesis of piglet's diarrhea worldwide (Katsuda *et al.,* 2006; Halaihel *et al.,* 2010), and are often seen in association with other enteropathogens (e.g. ETEC; Melin *et al.,* 2004; Kim *et al.,* 2010a), suggesting that they provoke damage on the epithelium that can alter the binding sites on enterocytes, favoring gut colonization by other enteropathogens (Melin *et al.,* 2004).

In suckling piglets *I. suis* occurred with much lower frequency (8.9%) than previously reported (44.7%) in the Havana province by Koudela *et al.* (1989); the housing improvement carried out in Cuban piggeries during last years could be a factor responsible for this difference. Like in the present survey, in Australia the highest frequency of this parasite was found in suckling piglets (Johnson *et al.,* 2008). Four out of 6 (66.6%) *I. suis* positive pigs were also infected with ETEC, similar results (45.7%) were shown by Chae *et al.* (1998) in South Korea. Choi *et al.* (2003) stated that isosporosis could promote intestinal colonization by ETEC due to an increase of glycoconjugates on the jejunal enterocytes.

The 10% occurrence of *C. parvum* found here is higher than the 2.1% reported in Havana province, Cuba, by Cabrera and García (1985). However, our higher prevalence could be due to the high sensitivity of the identification procedure used here (DAS-ELISA) compared with flotation and staining methods. The prevalence of *C. parvum* in young pigs has ranged from 1.4% to 71% worldwide (Izumiyama *et al.,* 2001; Wieler *et al.,* 2001; Yu and Seo, 2004; Maddox-Hyttel *et al.,* 2006). Experimental infections have confirmed the pathogenicity of *C. parvum* in pigs (Enemark *et al.,* 2003ab), and field studies have displayed a positive correlation of *Cryptosporidium* with swine diarrhea (Mišic *et al.,* 2003; Hamnes *et al.,* 2007). Probably, *C. parvum* acts often in concert with other enteropathogens to induce or exacerbate diarrhea; in that context it is interesting to remark that a mixed infection of rotavirus and *Cryptosporidium* aggravates diarrhea (Enemark *et al.,* 2003a), this mixed condition was not identified in the present study, but 55.5% of *C. parvum* infections were found to be combined with other enteropathogens.

That 25% of enteropathogen positive pigs had mixed infections confirms that in order to control diarrhea on a piggery, a thorough differential diagnosis has to be performed as recommended by Katsuda *et al.* (2006) and Straw *et al.* (2006). However, worldwide many studies assessing the

infectious etiology of swine diarrhea have only analyzed single etiologies (Chae *et al.,* 2000; Hong *et al.,* 2006). The differential identification of swine enteropathogens should be performed during *in vivo* experiments on swine diarrhea in order to accurately know the influence, or not, of certain pathogens on results (Baba and Gaafar, 1985; Choi *et al.,* 2003; Jensen *et al.,* 2006; Jung *et al.,* 2008). For instance, Lecce *et al*. (1982) reported that diarrhea caused by a rotavirus-ETEC mixed infection is more severe than the one caused by rotavirus or ETEC infection alone.

Enteropathogens were not detected in 46.7% of pigs during this study. This could be due to 1/tests sensitivity; 2/non-infectious factors (Straw *et al.,* 2006); 3/enteropathogens not-tested in the present study like other groups of rotaviruses (Kim *et al.,* 2010a), *Clostridium difficile* or PRRSV (Yaeger *et al.,* 2002); 4/not testing of some virulence factors (i.e. EAST-I, AIDA-I, PAA; Lee *et al.,* 2008); 5/insufficient recovery of the intestinal mucosa, such as the villous structure, after pathogen elimination; 6/ testing during the prepatent period of parasites where parasites are not yet detectable by general parasitological methods (Karamon *et al.,* 2007; Johnson *et al.,* 2008).

In conclusion, the study reported herein demonstrates that several enteropathogens are associated with porcine pre- and post-weaning diarrhea in the Villa Clara province, Cuba, of which ETEC is the most frequent. The improvement of swine enteropathogens identification, as well as the implementation of prevention and control programs for piglet's diarrhea which consider all potentially associated enteropathogens, particularly ETEC, might have a positive impact on the performance of young pigs in Cuba.

2.6 Acknowledgments

This study was funded by an Interuniversity Cooperation Program among Vlaams Interuniversitaire Raad (Belgium) and Universidad Central "Marta Abreu" de Las Villas (Cuba), specifically by the sub-project 5: *Improving the quality of graduate and postgraduate education and research programs in plant and animal sciences.* The Cuban Company for Pork Production and the Cuban Institute of Veterinary Medicine are thanked for supporting this survey. Are specially thanked the veterinarians from the sampled piggeries and colleagues Dr. Ania Gonzalez, DVM Einar Artiles, DVM Alberto Rodríguez, and DVM Lester Rodríguez for their help during sampling.

Chapter 3

Antibiotic resistance and genetic relatedness among pathogenic E. coli *isolated from intestinal contents of diarrheic piglets in Villa Clara province, Cuba.*

Based on:

de la Fé Rodríguez PY, Ndemi Kiiru J, Maroto Martin LO, Cruz Muñoz E, Butaye P, Cox E, Goddeeris BM, 2012. Characterization and clonal grouping of pathogenic *Escherichia coli* isolated from intestinal contents of diarrheic piglets in Villa Clara province, Cuba, according to their antibiotic resistance and ERIC-PCR profiles. Veterinary Microbiology, 154:425-8.

3.1 Abstract

This survey was undertaken to determine the insufficiently known antibiotic resistance and genetic relatedness among pathogenic *E. coli* associated with piglet's diarrhea in Villa Clara province, Cuba. Twenty six *E. coli* belonging to 10 virotypes, and isolated from intestinal contents of 90 piglets suffering from pre- or post-weaning diarrhea in 6 large indoor piggeries, were tested by the Kirby-Bauer disk diffusion method for antibiotic resistance, and by ERIC-PCR (employing primers ERIC1R: 5′-ATG-TAA-GCT-CCT-GGG-GAT-TCA-C-3′ and ERIC2F: 5′-AAG-TAA-GTG-ACT-GGG-GTG-AGC-G-3′) for genetic relatedness. The higher resistance rates were displayed to antibiotics of old generations which have been traditionally administered under uncontrolled prescriptions in Cuban piggeries. A significantly highest ($p<0.05$) resistance rate was found to tetracycline (69%); also, the resistance to ampicillin (54%), sulphonamide compounds (50%) and kanamycin (50%) were high, and 65% of isolates were multi-drug resistant. The ERIC-PCR revealed a high degree of polymorphism in the *E. coli* DNA sequences and relatedness among $F4^+/STa^+/STb^+$ or $F18^+/LT^+/STb^+$ virotypes isolated from different piggeries, and among STb^+, STa^+/STb^+, $F4^+/STa^+/STb^+$ or $F18^+/STx2e^+$ isolates from the same piggery. The antibiotic resistance and ERIC-PCR profiling were of help when assessing similarity of isolates: 17 clonal groups were identified. The results of this survey confirm that genetically diverse as well as genetically related pathogenic *E. coli* highly susceptible to many antibiotics (i.e. nalidixic acid, ciprofloxacin, gentamicin, amikacin, chloramphenicol, cephalosporins, amoxicilline-clavulanic acid, and trimethoprim), are associated with piglet's diarrhea in Villa Clara province, Cuba. These findings might be implemented during surveillance and control programs of swine colibacillosis in Cuba.

3.2 Introduction

Enteric infections with ETEC or VTEC are important causes of diarrhea or edema disease in pigs, which cause huge economic losses to the swine industry due to mortality, costs of medication, and growth retardation (Francis, 1999; Lazo *et al.,* 2005). Studies aimed on the differential identification of enteropathogens have reported ETEC, alone or as part of mixed infections, as a commonly associated pathogen with piglet's diarrhea worldwide (Wieler *et al.,* 2001; Nagy and Bilkei, 2003; Katsuda *et al.,* 2006; Hong *et al.,* 2006).

The antibiotic resistance profiling and genetic relatedness study among enteropathogenic *E. coli* are important contributions to the epidemiological characterization and control of swine diarrhea (Osek, 2000; Maynard *et al.,* 2003; Hariharan *et al.,* 2004; Lee *et al.,* 2009). However, in Cuba, where diarrheic diseases are responsible for 31% and 37% of the total piglet's mortality during the pre- and post-weaning periods, respectively (Cabrera and García, 2009), the identification of enteropathogens is limited, and the antibiotic resistance of porcine enterobacteria has not been surveyed (Barrera *et al.,* 2005; Lazo *et al.,* 2005; Blanco *et al.,* 2006).

Many DNA-based tests have been applied to reveal genetic relatedness among *Enterobacteriaceae* (e.g. PFGE, REP-PCR, BOX-PCR, and ERIC-PCR; Versalovic *et al.,* 1991; Cesaris *et al.,* 2007; Duan *et al.,* 2009). Enterobacterial repetitive intergenic consensus (ERIC) sequences are highly conserved at the nucleotide sequence level, and they constitute an effective marker when identifying clonal variability of isolates because their chromosomal locations and numbers differ among *Enterobacteriaceae* clones (Versalovic *et al.,* 1991). The ERIC-PCR fingerprinting analysis has been proved to be discriminatory during molecular differentiation of *E. coli* of porcine origin, and provides a better knowledge of their genetic relatedness and clonal origin (Osek, 2000; Namvar and Warriner, 2006; Yuan *et al.,* 2010). Additionally, Dias *et al.* (2009) observed that the circulating clonal composition of pathogenic *E. coli* tested by ERIC-PCR correlates with drug resistance prevalence.

Then, the aim of this study was to determine the antibiotic resistance profile and genetic relatedness among pathogenic *E. coli* isolated from piglets suffering from pre- or post-weaning diarrhea in Villa Clara province, Cuba, as an important contribution to the surveillance and control programs of swine colibacillosis.

3.3 Materials and methods

Animals and E. coli *isolates*

All pathogenic *E. coli* surveyed herein were isolated in the study discussed in Chapter 2, which was aimed at identifying the frequency of enteropathogens in intestinal contents of pigs suffering

from pre-weaning (n = 45) or post-weaning diarrhea (n = 45) in six large indoor piggeries (>500 sows per piggery) located in Villa Clara province, Cuba.

Antibiotic susceptibility test

The *E. coli* isolates were tested by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar according to the recommendations of the National Committee for Clinical Laboratory Standards (NCCLS; M2-A7, M100-S10). For this test were selected disks containing antibiotics traditionally administered in Cuban piggeries (i.e. tetracycline-30 µg, sulphonamide compounds-300 µg, ampicillin-10 µg, gentamicin-10 µg, and kanamycin-30 µg; Oxoid), as well as disks containing antibiotics less or practically never administered (i.e. trimethoprim-5 µg, trimethoprim:sulphamethoxazole/1:19-25µg, chloramphenicol-30 µg, amoxicilline-clavulanic acid-30 µg, ciprofloxacin-5 µg, nalidixic acid-30 µg, cefotaxime-30 µg, cefazolin-30 µg, and amikacin-30 µg). The isolates were classified in susceptible, intermediate resistant or resistant according to the inhibition zone diameter showed to each antibiotic and the breakpoints refered by the NCCLS. *E. coli* ATCC 25922 was used as susceptible control, and *Staphylococcus aureus* ATCC 3359 was the resistant control for most of antibiotics. Resistance rates were calculated by dividing the number of resistant and intermediate-resistant isolates by the total number of isolates. Comparison of resistance rates among antibiotics was performed by the chi-square test (χ^2) in StatGraphics Plus Version 5.0 ($p<0.05$).

Genetic relatedness test

Genetic relatedness was determined by the ERIC-PCR fingerprinting assay (Versalovic *et al.,* 1991). The ERIC-PCR reaction (25 µl) contained 3 µl of template DNA previously obtained (Chapter 2), 25 pmol of each primer (ERIC1R: 5′-ATG-TAA-GCT-CCT-GGG-GAT-TCA-C-3′ and ERIC2F: 5′-AAG-TAA-GTG-ACT-GGG-GTG-AGC-G-3′; Versalovic *et al.,* 1991), 600 µM of dNTPs (Roche Diagnostics GmbH), 1 U of SuperTaq (HT Biotechnology Ltd.), 2.5 µl of 10x SuperTaq buffer, and 2 mM of $MgCl_2$ (Promega). The PCR amplifications were performed in a thermal cycler PTC-100™ (MJ Research Inc.) by pre-denaturation of the mixture at 95°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and a long extension at 68°C for 8 min, the final extension was at 65°C for 13 min. Eight µl of the PCR products were electrophoretically separated in a 1.5% agarose gel in 1xTAE for 40 min at 80 V plus 1.5 h at 90 V. The DNA bands were stained with ethidium bromide for 30 min and visualized under UV light; the gel image capture was helped by the software Quantity-One (BIO-RAD Laboratories Inc.).The *E. coli* isolates were considered to belong to a single clonal group if they showed a highly similar electrophoretic banding pattern for the ERIC-PCR

products as previously described (Osek, 2000; Dias *et al.,* 2009; Bert *et al.,* 2010), and if they had a similar antibiotic resistance profile.

3.4 Results

Antibiotic susceptibility

Only 4 out of 26 *E. coli* were susceptible to all tested antibiotics. A significantly highest ($p<0.05$) resistance rate (69%) was detected to tetracycline. The resistance rates to ampicillin (54%), sulphonamide compounds (50%), and kanamycin (50%) were also high. Significantly lower ($p<0.05$) resistance rates were found for chloramphenicol (12%), nalidixic acid (12%), and gentamicin (4%). All isolates were susceptible to the rest of tested antibiotics (i.e. trimethoprim, trimethoprim:sulphamethoxazole, amoxicilline-clavulanic acid, ciprofloxacin, cefotaxime, cefazolin, and amikacin). Multi-drug resistance was present in 17 out of 26 (65%) isolates: 4 (15%) were resistant to two antibiotics, 3 (12%) to three, 8 (31%) to four, and 1 (4%) to five and six.

$F18^+/LT^+/STb^+$ and $F4^+/STa^+/STb^+$ isolates were the most multi-resistant among fimbriated *E. coli*. Chloramphenicol resistance was mostly found in all $F18^+/LT^+/STb^+$ *E. coli*, and VTEC ($F18^+/STx2e^+$) were susceptible to all antibiotics, except to sulphonamide compounds (Fig. 1).

Genetic relatedness and clonal groups

The ERIC-PCR revealed that pathogenic *E. coli* associated with porcine pre- and post-weaning diarrhea in Villa Clara province, Cuba, have a high degree of polymorphism in their DNA sequences (Fig. 1), suggesting that most of them are from different clones.

Seventeen clonal groups were distinguished among 26 *E. coli* tested herein. Overall, the genetic profiling corresponded with the antibiotic resistance profile of the isolates and with the virotype where to they belonged (Fig. 1).

The 3 $F18^+$ VTEC isolated from piggery D belonged to a homogeneous group (XII) as they generated almost an identical ERIC fingerprinting profile and had a similar antibiotic resistance profile. The 2 $F18^+/LT^+/STb^+$ *E. coli*, which were isolated from different piggeries (E and F), had also similar ERIC-PCR and antibiotic resistance, they belonged to group XI.

Four $F4^+/STa^+/STb^+$ *E. coli* isolated from suckling and weaned pigs in piggeries E and C appeared to belong to a single clonal group (XV). Although having a similar antibiotic resistance, the banding pattern of the $F4^+/^-$ *E. coli* isolated in piggery C was slightly different from the ones of $F4^+/STa^+/STb^+$ *E. coli*. There appeared a genetic diversity among the 8 STa^+/STb^+ *E. coli*, which were categorized in 6 clonal groups. The 2 STb^+ *E. coli* isolates from piggery A, which did not have antibiotic resistance, had identical genetic profiles.

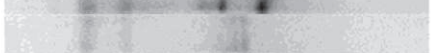
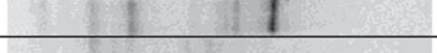
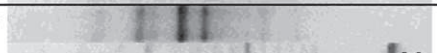
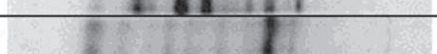
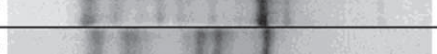
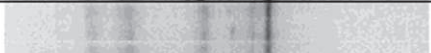
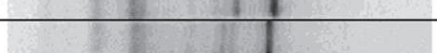
Virotype	Piggery/pig	ERIC-PCR profile	Antibiotic resistance profile	Group
STa$^+$	D/s		Amp-Sul	I
STb$^+$	F/w		Tet-Amp-Sul-Kan-Chl-Gen	II
	A/w		None	III
	A/s		None	III
STa$^+$/STb$^+$	F/s		Tet-Amp-Kan	IV
	D/w		Tet-Kan-Nal	V
	D/w		Tet-Amp-Kan-Nal	V
	D/s		Tet-Kan	VI
	D/s		Kan	VII
				
	B/s		Tet	VIII
	B/s		Tet	VIII
	B/s		Tet-Amp	IX
LT$^+$/STb$^+$	C/s		Tet-Amp-Sul-Kan	X
F18$^+$/LT$^+$/STb$^+$	E/w		Tet-Amp-Sul-Chl	XI
				
	F/w		Tet-Sul-Kan-Chl-Nal	XI
F18$^+$/STx2e$^+$	D/w		Sul	XII
	D/w		None	XII
	D/w		Sul	XII
F6$^+$/STa$^+$	D/w		None	XIII
				
F4$^+$	C/w		Tet-Amp-Sul-Kan	XIV
F4$^+$/STa$^+$/STb$^+$	E/s		Tet-Amp-Sul-Kan	XV
	E/s		Tet-Amp-Sul	XV
	C/s		Tet-Amp-Sul-Kan	XV
	C/w		Tet-Amp-Sul-Kan	XV
	C/w		Tet-Amp-Sul-Kan	XVI
F5$^+$/F41$^+$/STa$^+$	E/s		Tet-Amp	XVII
S. Newport	F/w		Amp-Sul	S-I
	A/w		Sul	S-I
				

Figure 1. Clonal groups of pathogenic *E. coli* associated with piglet's diarrhea in Villa Clara province, Cuba, according to their virotype, ERIC-PCR and antibiotic resistance profiles.

S, suckling piglet; W, weaned pig; M, 100bp marker run in the same gel of ERIC-PCR products visualized on its top; Amp, ampicillin; Sul, sulphonamide compounds; Tet, tetracycline; Kan, kanamycin; Chl, chloramphenicol; Gen, gentamicin; Nal, nalidixic acid; *S*. Newport, *Salmonella enterica* subspecies *enterica* serotype Newport isolated from intestinal contents of weaned pigs and analyzed by the ERIC-PCR and antibiotic susceptibility tests employed for *E. coli.*

3.5 Discussion

For the last 20 years, specific reports or epidemiological studies regarding porcine enteropathogens in Cuba have been scarce (Fuentes *et al.,* 2001; Barrera *et al.,* 2005; Lazo *et al.,* 2005; Blanco *et al.,* 2006). A recent survey by de la Fé Rodríguez *et al.* (2011) reported a high serological prevalence of $F4^+$ and $F18^+$ *E. coli* in the Cuban swine herd, indicating that these pathogenic bacteria are widespread as potential etiologic agents of colibacillosis; also, ETEC was found to be the most associated enteropathogen with piglet's diarrhea in Villa Clara province, Cuba (25.6% frequency; Chapter 2). Additionally, the present survey determined the antibiotic resistance profile and genetic relatedness among pathogenic *E. coli* associated with porcine pre- and post-weaning diarrhea in Villa Clara province.

A high resistance rate to the traditionally administered antibiotics and a high genetic diversity among pathogenic *E. coli* were observed. The genetic profiling by ERIC-PCR enabled to categorize 26 pathogenic *E. coli* into 17 clusters with a more or less close relationship with the different antibiotic resistance profiles, taking into consideration the virotype of the isolates. Such integral studies have been rarely reported (Lee *et al.,* 2009; Thorsteinsdottir *et al.,* 2010).

Different from reports showing high resistance of *Enterobacteriaceae* to a wide range of antibiotics worldwide (Hendriksen *et al.,* 2008; Knezevic and Petrovic, 2008; Smet *et al.,* 2009; Tian *et al.,* 2009), herein were exclusively displayed high resistance rates to antibiotics routinely used for treating diarrhea and other diseases in Cuban piggeries during last years (i.e. tetracycline, ampicillin, sulphonamides, and kanamycin). Vieira *et al.* (2009) stated that there is an association between tetracycline treatment incidence rate in a herd and the probability of isolating a tetracycline resistant *E. coli* from the intestinal content of pigs. The resistance rate to gentamicin was low (4%) despite being frequently administered in Cuba. Except for chloramphenicol and nalidixic acid, for which 12% resistance rates were displayed, all isolates were susceptible to antibiotics less or practically never administered in Cuban piggeries (i.e. trimethoprim, trimethoprim:sulphamethoxazole, amoxicilline-clavulanic acid, ciprofloxacin, cefotaxime, cefazolin, and amikacin). Maynard *et al.* (2003) concluded that the genes behind phenotypic resistance are not static but are rather in a state of flux driven by various selection forces such as the use of specific antimicrobials, statement that may also justify the high susceptibility of *E. coli* to antibiotics less or practically never administered in Cuban piggeries found here.

In the near future, as recommended by Hendriksen *et al.* (2008) and Harada *et al.* (2008), data from antibiotic resistance surveillance in every geographic area or piggery have to be taken into account by the veterinary authorities when choosing antimicrobials for controlling swine colibacillosis in Cuba; but first, the specific identification and characterization of enteropathogens

have to be improved in the regional Veterinary Diagnostic Laboratories in order to avoid the uncontrolled antibiotic indication for treating diarrhea, which is mostly diagnosed by only clinical or macropathological examinations.

It is interesting that similar to this report, Maynard *et al.* (2003), Hariharan *et al.* (2004), Varga *et al.* (2008), and Akwar *et al.* (2008) found high resistance rates to tetracycline, ampicillin, and sulphonamide compounds, as well as high susceptibility to gentamicin and cephalosporins in porcine enteric *E. coli* from Canada. In Cuba, thousands of breeding stock pigs have been imported from Canada during last decade, and they could be the source of pathogens of non-obligatory declaration like *Enterobacteriaceae* carrying genes encoding drug resistance, but other epidemiological studies are needed to prove it. Previous information about antibiotic resistance of porcine *E. coli* from Cuba is not available (Blanco *et al.,* 2006).

The ERIC-PCR was applied for the first time to Cuban isolates of porcine pathogenic *E. coli* and was useful for their clonal and epidemiological discrimination. Among herds, $F4^+/STa^+/STb^+$ isolates from piggeries E and C, as well as $F18^+/LT^+/STb^+$ isolates from piggeries E and F, were found to be clonally related. The frequent trading of breeding stock pigs among piggeries and other ways affecting piggery's biosafety could lead the spreading of pathogenic *E. coli* in Villa Clara province. Clonal relations were also found inside herds for STb^+ isolates in piggery A, for STa^+/STb^+ isolates in piggeries D and B, for $F18^+/STx2e^+$ isolates in piggery D, and for $F4^+/STa^+/STb^+$ isolates in piggeries E and C. ERIC-PCR can be applied for identifying bacterial species, types, or isolates (Yuan *et al.,* 2010), and is an accurate and reliable technique for studying the molecular epidemiology of pathogenic *Enterobacteriaceae* (Dalla-Costa *et al.,* 1998; Manges *et al.,* 2004; Ramchandani *et al.,* 2005; Ibenyassine *et al.,* 2006; Jurkovič *et al.,* 2007) and other bacteria like *Pasteurella* (Saxena *et al.,* 2005). ERIC-PCR profiling of *E. coli* enabled also to identify contamination sites in a high capacity pork slaughter line (Namvar and Warriner, 2006).

In conclusion, the antibiotic therapy routinely employed in Cuba for controlling swine colibacillosis should be changed as most of *E. coli* tested herein were resistant to all commonly administered antibiotics, except to gentamicin. The introduction of other drugs for which ETEC showed high susceptibility (e.g. nalidixic acid, ciprofloxacin, gentamicin, or trimethoprim) appears advantageous; but first, it is necessary that the provincial Veterinary Diagnostic Laboratories improve the identification of pathogenic *E. coli* as well as the antibiotic resistance surveillance. The analysis on genetic diversity by ERIC-PCR demonstrated a clonal relationship among pathogenic *E. coli* carrying the same virulence factors and similar antibiotic resistance. The high genetic diversity of porcine enteropathogenic *E. coli* found in this survey was previously demonstrated in Cuba, when Blanco *et al.* (2006) described 21 distinct restriction patterns in 24 pathogenic *E. coli* by PFGE. As

both studies have been only performed in Central Cuba, the application of these epidemiological researches throughout the whole country might identify the *E. coli* clones which are most associated with clinical disease, and will contribute to the surveillance, prevention, and control of swine colibacillosis.

3.6 Acknowledgments

This study was funded by an IUC program between VLIR-UOS (Belgium) and Universidad Central "Marta Abreu" de Las Villas (Cuba). DVM Miguel Angel Arce, DVM Einar Artiles, and Ir. René Cupull are specially thanked for their assistance during antibiotic resistance testing.

Chapter 4

High prevalence of F4^{+} and F18^{+} E. coli *in Cuban piggeries as determined by serological survey*

Based on:

de la Fé Rodríguez PY, Coddens A, Del Fava E, Cortiñas Abrahantes J, Shkedy Z, Maroto Martin LO, Cruz Muñoz E, Duchateau L, Cox E, Goddeeris BM, 2011. High prevalence of F4^{+} and F18^{+} *Escherichia coli* in Cuban piggeries as determined by serological survey. Tropical Animal Health and Production, 43:937-46.

4.1 Abstract

Little information is available on the prevalence of swine enteropathogens in Cuba where diarrheic diseases are responsible for 31% and 37% of the total mortality during the neonatal and post-weaning periods. $F4^+$ and $F18^+$ ETEC and $F18^+$ VTEC induce diarrhea and edema disease in pigs, but their distribution has never been thoroughly studied in the Cuban swine population. Therefore, the present study estimated the prevalence of F4- and F18-specific antibodies in sera of 1044 6-months old gilts distributed in 34 piggeries spread over the Cuban territory. For the data analysis, which included the optical density of individual samples tested by ELISA, random-effects models and a mixture model in R (package "mixAK"; Komárek, 2009) were fitted. Low, moderate, and high levels of F4-specific antibodies were found in 67.6%, 26.8%, and 5.6% of the gilts, while 66.4% and 33.6% of gilts showed low and high levels of F18-specific antibodies. Hereby, we show that $F4^+$ and $F18^+$ *E. coli* are highly prevalent as potential enteropathogens in Cuban piggeries.

4.2 Introduction

$F4^+$ and $F18^+$ ETEC are common causes of diarrhea in suckling and recently weaned piglets worldwide. $F18^+$ VTEC causes edema disease, which is characterized by edema of the eyelids, ears, stomach, and colon as well as dyspnea, neurological symptoms, and sudden death (Oanh *et al.,* 2010). Zhang *et al.* (2007) implicated F4 and F18 as the major fimbrial antigen types among problems of ETEC and VTEC infections.

The pathogenesis of colibacillosis caused by $F4^+$ and $F18^+$ *E. coli* is well-known. The fimbriae mediate the adhesion of bacteria to specific receptors present on the surface of the enterocytes of the small intestine, leading to bacterial colonization and subsequent production of enterotoxins (LT, STa, and/or STb) which change the water and electrolyte balance across the mucosa leading to diarrhea. The edema disease is caused by the systemic vascular damage provoked by verocytotoxin (Stx2e; Fairbrother, 2006; Fairbrother and Gyles, 2006).

Swine colibacillosis causes losses due to mortality, costs of medication, and growth retardation, which have a significant negative impact on the economic feasibility of the swine industry (Lazo *et al.,* 2005; Blanco *et al.,* 2006; Tiels *et al.,* 2008). Housing and management conditions (e.g. hygiene, environment temperature, intake of maternal antibodies, and feeding), vaccination, and surveillance of antibiotic resistance are crucial in prevention of porcine neonatal and post-weaning diarrhea (Fairbrother *et al.,* 2005; Fairbrother, 2006).

In 2008, the average survival rate at weaning (number of weaned pigs/number of born pigs) on swine farms of the Cuban Company for Pork Production was as low as 70%. In Cuban piggeries, a specific etiologic diagnosis of the swine enteric pathogens is not always possible to perform. As a result, there is little information available on the prevalence of enteropathogens in Cuba where diarrheic diseases are responsible for 31% and 37% of the total mortality during the pre- and post-weaning periods, respectively (Cabrera and García, 2009).

F4- and F18-specific antibodies can be induced by parenteral vaccination, but also by an $F4^+$ and $F18^+$ ETEC/VTEC infection, respectively (Van den Broeck *et al.,* 1999a; Verdonck *et al.,* 2002). Vaccinations against F4 or F18 have not been applied in Cuba during the last decade; therefore, F4- or F18-specific serum antibodies in Cuban pigs will reflect the presence and spread of $F4^+$ ETEC or $F18^+$ ETEC/VTEC.

4.3 Materials and methods

Survey design

This survey was performed on serum samples of replacement gilts (6 months old) originally collected by the Cuban Veterinary Diagnostic Laboratories for *Leptospira* and *Brucella* routine screening. Based on the experience of veterinary practitioners and a few reports of $F4^+$ and $F18^+$ *E.*

coli in Cuba, a prevalence between 60% and 90% of F4- and F18-specific antibodies was expected. Because a similar serosurvey has not been previously performed in Cuba, a 60% expected prevalence was selected as it gives a higher sample size (n = 1,025 versus n = 385 for the 90% expected prevalence). A 95% confidence interval and a low 3% desired absolute precision were fixed, resulting in a small confidence interval for theoretically infinite populations (Thrusfield, 1997; Naing *et al.,* 2006).

During the period between February and June 2008, 1,044 (19 more than planned) serum samples from farms of the Cuban Company for Pork Production (GRUPOR) were collected. In practice, they were distributed in 34 out of 94 piggeries of the company located in 10 out of 14 provinces all over the three Cuban regions (Fig. 1).

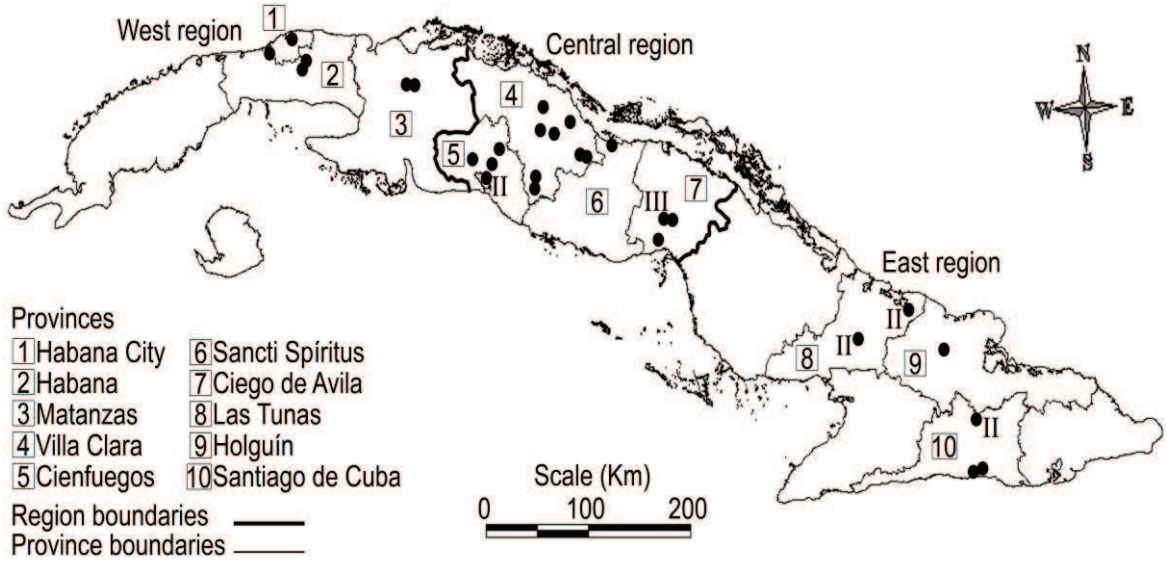


Figure 1. Map of Cuba showing the geographic location of the 34 piggeries (*black dots*) from where the prevalence of F4- and F18-specific antibodies were estimated. *Roman numerals* indicate the number of piggeries with similar location.

Collection, transport, and treatment of blood serum samples

Blood was collected from the orbital venous sinus (Fig. 2), and serum was obtained after 3 h incubation at environment temperatures (32-35°C) and stored at 4°C. Subsequently, centrifugation and heat inactivation were performed, followed by treatment with kaolin to remove nonspecific serum inhibitors. One volume of serum was mixed with four volumes of kaolin suspension [25% (*w/v*) in PBS (150 mM, pH 7.4)], and incubated at room temperature for 30 min. The serum was recovered after centrifugation at 14,000×*g* for 10 min and was stored at -20°C.

Figure 2. Puncture of the orbital venous sinus helped by a California needle in a pig, which is traditionally employed for blood collection or even for fluid-therapy in Cuba.

Purification of F4 and F18 fimbriae

The protocol was similar to the mechanical shearing described by Verdonck *et al.* (2002). F4 and F18 fimbriae were obtained from the *E. coli* strains IMM-01 (O149:K91/F4ac$^+$/LT$^+$/STb$^+$) and 107/86 (O139:K12:H1/F18ab$^+$/Stx2e$^+$), respectively. Briefly, the *E. coli* strains were grown in TSB (Oxoid) at 37°C and 85 rpm overnight. Next, bacteria were collected by centrifugation (2851×*g*, 35 min, 4°C) and the pellet was resuspended and washed with PBS. Bacterial suspension was mixed on ice using an Ultra Turrax (IKA, Labortechnik) during 20 min. Afterwards, the bacterial cell debris was pelleted by centrifugation (26892×*g*, 40 min, 4°C, two times) and the fimbriae, contained in the supernatant, were precipitated with ammonium sulphate (at 40% saturation for F4 and at 20% saturation for F18, overnight, 4°C). The precipitated fimbrial suspension was centrifuged (1455×*g*, 30 min, 4°C), resuspended in PBS and dialyzed overnight (10 kDa cutoff) at 4°C against PBS. Finally, the quality of each fimbrial suspension was assessed by the Bicinchioninic acid reaction, 12% SDS-PAGE and Western Blot (Verdonk *et al*., 2002).

ELISA for detecting F4-specific immunoglobulins

An indirect double-antibody ELISA was performed as previously described (Tiels *et al.,* 2008). Briefly, a solution of 1 μg/ml in PBS of IMM01 anti-F4 MAb (Van der Stede *et al.,* 2002) was applied as a coating to a 96-well MaxiSorp plate and incubated for 2 h at 37°C. The plates were blocked overnight with 0.2% (*v/v*) Tween® 80 in PBS at 4°C. Then, the F4 antigen was added at 5 μg/ml in dilution buffer [0.2% (*v/v*) Tween® 20 + 3% (*w/v*) BSA in PBS]. Next, the serum samples were applied in duplicates at a fixed dilution of 1/15, followed by the addition of anti-swine IgG (H+L) HRP-conjugated supplemented with 1% mouse serum. The presence of the bound conjugate was revealed by adding ABTS, and the OD was spectrophotometrically measured at 405 nm after incubation for 15 min at 37°C. The plates were washed three times [0.2% (*v/v*) Tween® 20 in PBS] between each

incubation step, except for the coating and blocking steps. The incubation condition of antigens and antibody solutions was for 1 h at 37°C.

ELISA for detecting F18-specific immunoglobulins

The main difference from the F4 ELISA was the coating with F18 fimbriae (2 µg/ml in 50 mM sodium bicarbonate pH 9.4; Tiels *et al.,* 2008), while the blocking, washing, incubation, and detection steps were similar.

The negative and positive control sera employed in both ELISAs were provided by the Laboratory of Immunology, Department of Virology, Parasitology and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium.

Statistical analyses

For data standardization, the average OD of the negative control serum per ELISA plate was subtracted from the OD value of each sample. In order to obtain a more suitable classification of individual results, we decided not to use the commonly used diagnostic OD cutoff point (i.e., mean OD + 3*SD), but we modeled the homogenized data using a mixture of distribution.

It was assumed that pigs in the same location can be correlated; thus, three location variables (region, province, and piggery) were considered as grouping variables.

Let Y_{ijkl} be the vector of OD measurements, given the individual *i*, the region *j*, the province *k*, and the piggery *l*.

Firstly, we fit multilevel mixed-effects models, one for each data, using three levels of clustering, i.e. piggeries within provinces within regions, by means of SAS and R software, for the sake of comparison:

$$Y_{ijkl} = \mu + r_j + p_{jk} + f_{jkl} + \varepsilon_{ijkl}$$

, where the random effects *r, p,* and *f* and the residual error ε are independently normally distributed with mean 0 and variance equal to σ_j^2, σ_{jk}^2, σ_{jkl}^2 and σ_{ijkl}^2, respectively.

These models allow the mean OD estimation taking into account the additional variability due to the different clustering levels.

The correlation between F4 and F18 OD values was checked by the Pearson correlation coefficient with 95% confidence interval.

Secondly, in order to describe the overall distribution and to classify the individual OD values, we decided to fit the data using mixture models: the distribution of the data was fitted using a mixture of several Gaussian components, each of them representing a cluster of the data and having a

specific weight in the population. We fitted a series of Gaussian mixture models, increasing at each step the number of components up to 10. The analysis was done with the R package "mixAK" (Komárek, 2009), which estimates the mixture using Markov chain Monte Carlo (MCMC) methods. We used all the pigs pooled together, independently to their provenance: in this way, the classification of a pig in one of the components is only depending on its own OD value.

The choice of the optimal number of components was done using two selection criteria: the penalized expected deviance (PED; Plummer, 2008) and the difference in posterior deviances (Aitkin *et al.,* 2009). The PED penalizes the expected deviance $\overline{D(\theta)}$ with the "optimism" p_{opt} because of using the same data twice, once for estimation and once for prediction: thus, the smaller the PED, the better. The approach of Aitkin (Aitkin, 2009) starts from the fact that models with growing numbers of parameters are automatically penalized by the increasing dispersion of the posteriors in their parameters. Thus, the selection between two models is given by the difference between the posterior distributions of the deviances:

$$\left\{D_{1,2}^{(m)} = D_1^{(m)} - D_2^{(m)} : m = 1,\text{K}\ ,M\right\}$$

, where *M* is the length of the MCMC chain: we can derive from the posterior probability that Model 1 is better than Model 2,

$$\frac{1}{M}\sum_{m=1}^{M} I(D_{1,2}^{(m)} < 0)$$

, and derive 95% credible intervals for the difference in deviances.

4.4 Results

Exploratory data analysis: F4 OD data

After data standardization, only two out of 1,044 samples resulted negative in the F4 ELISA, and the OD of the positives fluctuated from 0.017 to 1.887. The mean OD value of all negative control serum was 0.212 (n=56, SD=0.040). The analysis of these "raw" data shows a high positivity and differences between individual samples, but the proper description of the whole population is difficult to make, justifying the following analyses.

Next, the median F4 OD value and the interquartile range (IQR) per piggery were determined (Fig. 2). The median OD values were shown to be different between piggeries. Seven out of 34 piggeries had a high median OD value for F4-specific antibodies (above the third quartile for the entire sample, i.e., 1.01), whereas 18 out of 34 showed moderate levels (the median OD value was between the first

and third quartile, i.e., 0.53 and 1.01). Nine piggeries had little amount of F4-specific antibodies as the median OD value was below the first quartile.

Four piggeries (out of 7) with high median OD value for F4-specific antibodies are located in the Central province Ciego de Avila (E1-E4). Similar results were obtained for B2 (Matanzas, west region), and for G3 and G4 (Santiago de Cuba, east region).

The piggeries with moderate OD values were found to be spread over the country.

The lower median OD values were found in piggeries A2 (Havana City), C8 (Villa Clara), D3-D5 (Cienfuegos), F1-F3 (Las Tunas), and G2 (Santiago de Cuba). In most of them, the upper whisker, which contain OD values within 1.5*IQR, was below the third quartile of the overall distribution, confirming the low level of F4-specific antibodies of sampled gilts.

Exploratory data analysis: F18 OD data

After data standardization, no negative values were found and the OD value of positives varied from 0.037 to 1.898. The mean OD value of all negative control serum was 0.106 (n=52, SD=0.020).

The distribution of F18 OD values between piggeries (Fig. 3) is more homogeneous compared to the F4 data as most piggeries (23 out of 34) had median F18 OD values between the first and the third quartiles of the overall distribution, which are equal to 0.51 and 1.01, respectively.

However, a high median OD value for F18-specific antibodies (above the third quartile, i.e., 1.01) was obtained in piggeries A1 (Havana City), C2 and C5 (Villa Clara), as well as E2 and E4 (Ciego de Avila).

Piggeries with the lowest median OD values are located in Matanzas (B1 and B2, west region), in Villa Clara (C1 and C8, central region) and in the eastern province Las Tunas (F2 and F3).

Random-effects models

Both multilevel models fitted in SAS and in R for F4 and F18 OD data confirm that most of the variance is residual or unexplained; there is some extra variation due to piggery, but very little due to province or region (Table 1). In particular, not all F4 variability is residual, and some heterogeneity can be ascribed to a smaller extent to the piggeries. Conversely, for F18, only the variance of the residual variability is significantly positive.

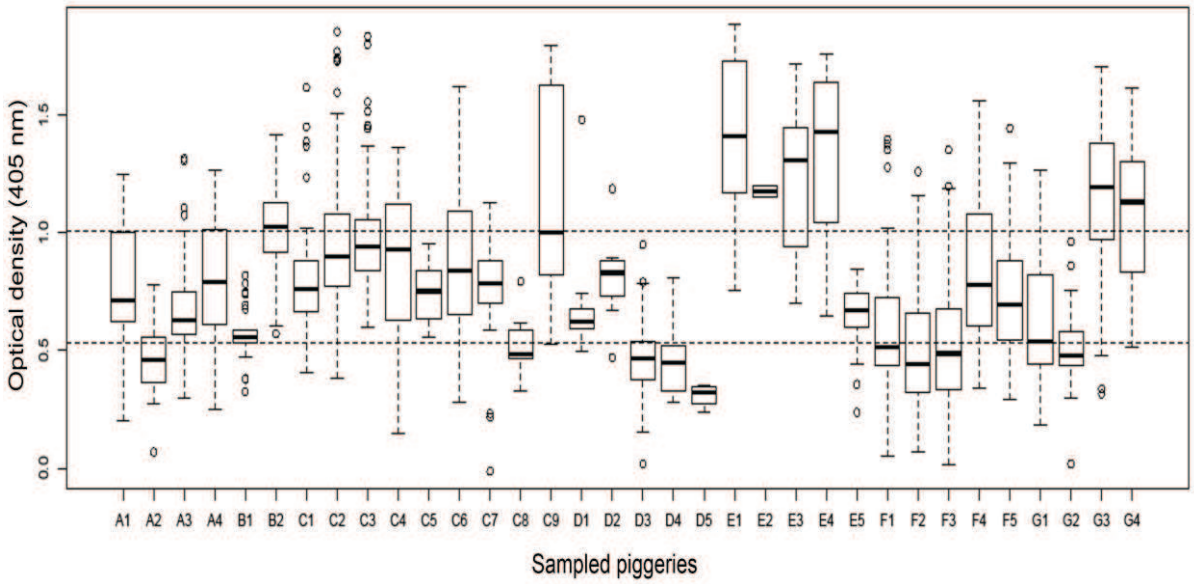


Figure 2. Box plot of the ELISA OD values for F4-specific antibodies between piggeries. The *boxes* contain OD values between the first and the third quartiles, and the *whiskers* contain OD values within 1.5*IQR. The *horizontal dashed lines* indicate the first and the third quartiles of the overall distribution. The *line inside the boxes* represents the median OD value. In the *x-axis*, piggeries are organized by province from West to East Cuba: *A1* and *A2* (Havana City), *A3* and *A4* (Havana), *B1* and *B2* (Matanzas), *C1-C8* (Villa Clara), *C9* (Sancti Spíritus), *D1-D5* (Cienfuegos), *E1-E5* (Ciego de Avila), *F1-F4* (Las Tunas), *F5* (Holguín), and *G1-G4* (Stgo de Cuba).

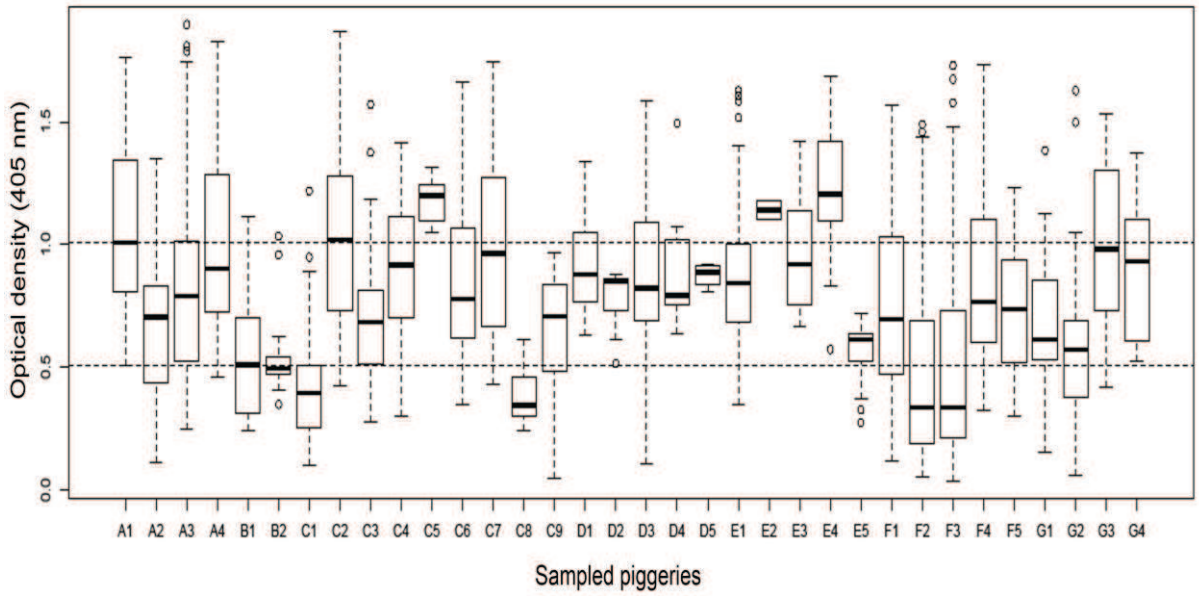


Figure 3. Box plot of the ELISA OD values for F18-specific antibodies between piggeries. The *boxes* contain OD values between the first and the third quartiles, and the *whiskers* contain OD values within 1.5*IQR. The *horizontal dashed lines* indicate the first and the third quartiles of the overall distribution. The *line inside the boxes* represents the median OD value. In the *x-axis*, piggeries are organized by province from West to East Cuba: *A1* and *A2* (Havana City), *A3* and *A4* (Havana), *B1* and *B2* (Matanzas), *C1-C8* (Villa Clara), *C9* (Sancti Spíritus), *D1-D5* (Cienfuegos), *E1-E5* (Ciego de Avila), *F1-F4* (Las Tunas), *F5* (Holguín), and *G1-G4* (Stgo de Cuba).

Table 1. Estimated mean (μ) and variance components (with standard errors) of the multilevel linear mixed model fixed for the F4 and F18 ELISA OD data using three levels of clustering (piggeries within provinces within regions).

Parameters	F4 OD data	F18 OD data
μ	0.80 (0.22)	0.80 (0.034)
Region	0	0
Province	0.021 (0.017)	0
Piggery	0.047 (0.0030)	0.040 (0.044)
Residual	0.076 (0.00086)	0.10 (<0.0001)

The study of Pearson correlation coefficient (r = 0.19) in R suggested that there is no correlation between F4 and F18 OD individual values, confirming the specificity of both ELISAs. Indeed, clear differences are observed when comparing the distribution of F4 and F18 OD values in the piggeries A1, B2, C5, C9, and E1 (Figs. 2 and 3).

Mixture modeling: F4 OD data

According to the PED, the best mixture has three components (PED=788), while the one with four components has a PED of 823. According to the difference in posterior deviances, the mixture with three components is much better than the mixture with two components, with $D_{1,2}^{(m)}$ =-19 and 95% credible interval (-29,-5) and posterior probability 0.99. Instead, we cannot confidently choose between the mixture with four components and with three, with $D_{1,2}^{(m)}$ =1 and 95% credible interval (-17, 18) and posterior probability 0.57. Thus, we choose the mixture with three components, because it almost fits in the same way as the one with four components, being at the same time more parsimonious in terms of parameters.

Looking at the estimates (Table 2), we can label the components in the following way: the component 1 contains the low OD values, and the components 2 and 3 stand for the moderate and high OD values, respectively. Then, all the observations are rather assigned to a particular component, instead of being spread among all the population.

Figure 4 presents the histogram of the data over imposing the estimated mixture with three components. We can notice that the biggest components, the ones for low and moderate OD values, are gathering the serum levels from 0 up to 1.5, while after that value, the third component is taking place and gathers the highest OD values.

For separating the three components, the mixture OD cutoff points were estimated at their intersection: the OD cutoff point between the lowly and the moderately positive pigs was 0.910, while between the moderately and highly positives was 1.551.

Then, we can accurately determine how individual cases could be classified according to their ELISA signal as the cutoff OD values were obtained from the overall distribution (Table 3).

The prevalence of gilts with low, moderate, and high levels of F4-specific antibodies was 67.6%, 26.8%, and 5.6% respectively.

Table 2. Mixture parameters estimate of the fixed Gaussian models for the F4 and F18 ELISA OD data in which are based the definition of anti-F4 and anti-F18 OD groups.

OD data	Components	π_k	μ_k	σ_k	OD groups
F4	1	0.599	0.614	0.224	Low
	2	0.349	1.036	0.283	Moderate
	3	0.052	1.683	0.117	High
F18	1	0.602	0.578	0.231	Low
	2	0.398	1.084	0.329	High

π_k , estimate for the mixing weights or prior probability; μ_k, mean; σ_k , standard deviation.

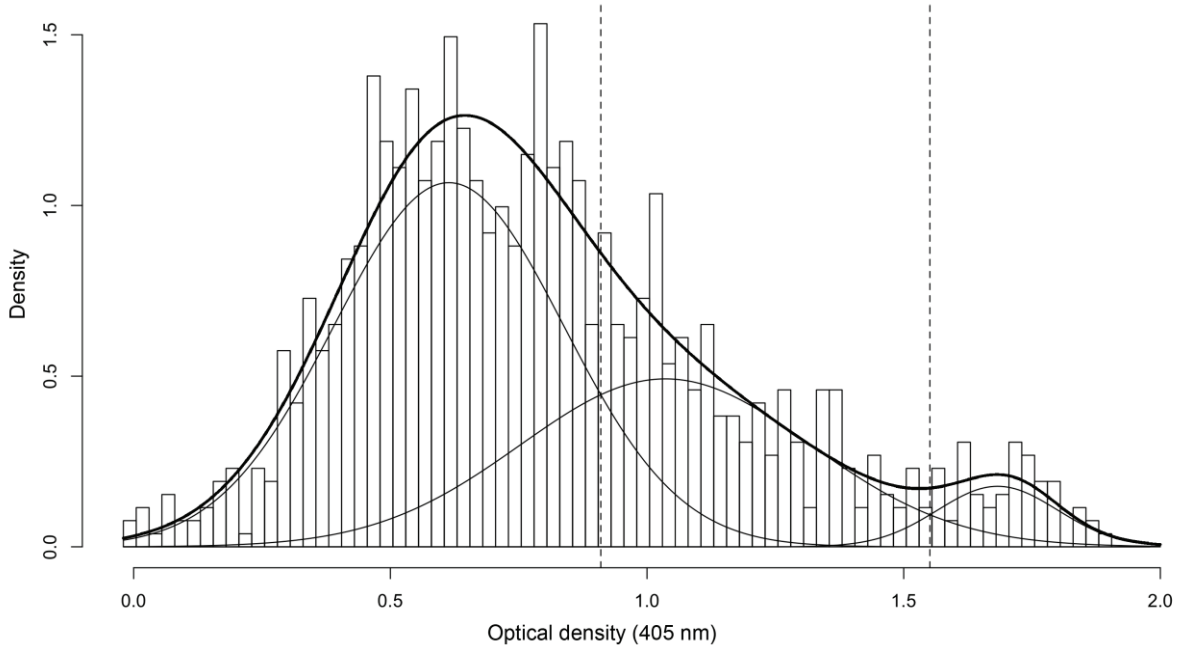


Figure 4. Histogram of the F4 ELISA OD data with the fixed three-component Gaussian mixture model (*thick curve*) overimposed, assessing the prevalence of F4-specific antibodies in Cuban swines. The *thin curves* show the three single components, and the *dashed lines* indicate the mixture OD cutoff points (0.910 and 1.551).

Table 3. Seroprevalence of F4-specific antibodies in Cuban piggeries according to the ELISA OD cutoff points calculated by the fixed three-component mixture model.

Piggeries	Number	Seroprevalence (%)		
		Low OD	Moderate OD	High OD
A1	17	71	29	0
A2	15	100	0	0
A3	76	91	9	0
A4	12	67	33	0
B1	29	100	0	0
B2	49	22	78	0
C1	31	77	19	3
C2	59	54	34	12
C3	73	44	52	4
C4	8	50	50	0
C5	14	93	7	0
C6	40	65	25	10
C7	28	79	21	0
C8	9	100	0	0
C9	26	35	35	31
D1	10	90	10	0
D2	9	89	11	0
D3	58	98	2	0
D4	8	100	0	0
D5	4	100	0	0
E1	48	8	50	42
E2	2	0	100	0
E3	8	25	63	13
E4	15	20	40	40
E5	27	100	0	0
F1	50	84	16	0
F2	52	87	13	0
F3	45	89	11	0
F4	60	63	35	2
F5	40	80	20	0
G1	30	83	17	0
G2	34	97	3	0
G3	46	22	65	13
G4	12	33	58	8
Total	1,044	67.6	26.8	5.6

Shaded lines represent the highly positive piggeries, where more than 50% of sows showed moderate or high OD values

Mixture modeling: F18 OD data

According to the PED, the best mixture has two components (PED=812), while the second best has six components (PED=852). Instead, according to the difference in posterior deviances, it is quite difficult to choose the best mixture. The posterior probabilities *P* (diff<0) increase as long as the number of components increases. For instance, if we compare the model with six and with two components, the posterior probability for the model with six groups is 0.70, with $D_{1,2}^{(m)}$ =5 and 95% credible interval (-16, 27), while if we compare the models with three and with two components, the posterior probability for the model with three groups is 0.58, with $D_{1,2}^{(m)}$ =2 and 95% credible interval (-16, 27). However, we notice that the mean of the difference is always positive and that the credible interval is more shifted toward the positive values. Given all this, we choose the mixture with two components, which is even indicated as the best one by the PED criterion.

Looking at the estimates (Table 2), the component 1 represents the group of gilts with the lower amount of F18-specific antibodies, whereas component 2 represents gilts with the higher amount of F18-specific antibodies.

Figure 5 presents the histogram of the data overimposing the estimated mixture with two components. The OD cutoff point determined by the intersection of both components was 0.894.

From the overall sample, we determined that 66.4% and 33.6% of gilts showed low and high OD values to F18-specific antibodies, respectively (Table 4).

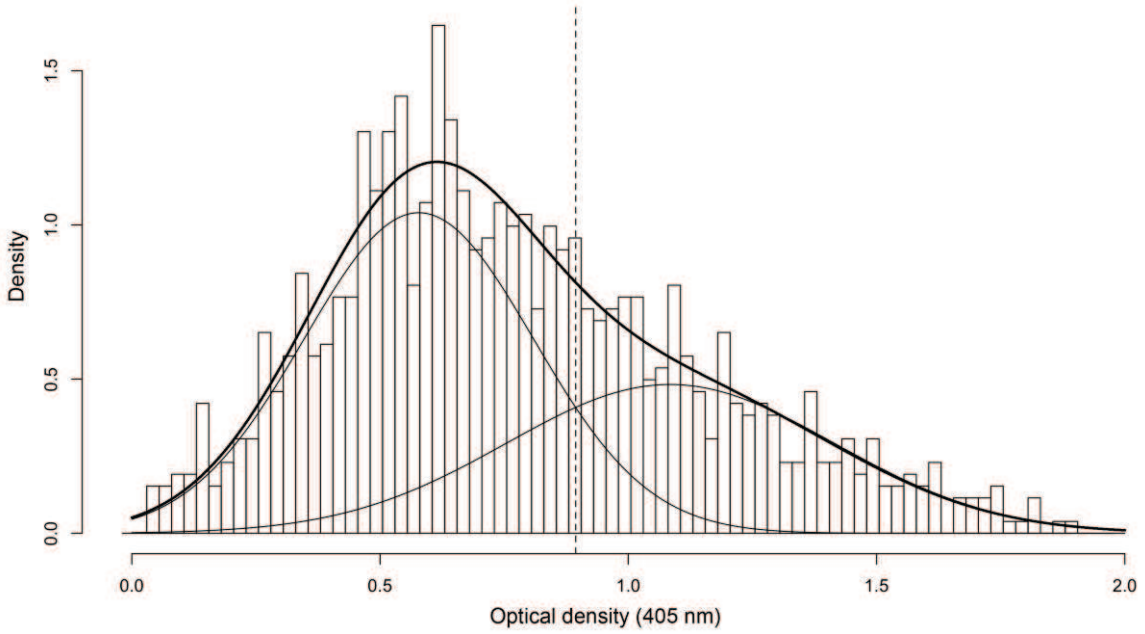


Figure 5. Histogram of the F18 ELISA OD data with the fixed two-component Gaussian mixture model (*thick curve*) overimposed, assessing the prevalence of F18-specific antibodies in Cuban swines. The *thin curves* show the two single components, and the *dashed line* indicates the mixture OD cutoff point (0.894).

Table 4. Seroprevalence of F18-specific antibodies in Cuban piggeries according to the ELISA OD cutoff point calculated by the fixed two-component mixture model.

Piggeries	Number	Seroprevalence (%)	
		Low OD	High OD
A1	17	41	59
A2	15	87	13
A3	76	63	37
A4	12	50	50
B1	29	79	21
B2	49	96	4
C1	31	94	6
C2	59	34	66
C3	73	85	15
C4	8	25	75
C5	14	0	100
C6	40	65	35
C7	28	46	54
C8	9	100	0
C9	26	77	23
D1	10	50	50
D2	9	100	0
D3	58	57	43
D4	8	63	38
D5	4	50	50
E1	48	54	46
E2	2	0	100
E3	8	50	50
E4	15	13	87
E5	27	100	0
F1	50	64	36
F2	52	83	17
F3	45	80	20
F4	60	65	35
F5	40	70	30
G1	30	80	20
G2	34	85	15
G3	46	39	61
G4	12	50	50
Total	1,044	66.4	33.6

Shaded lines represent the highly positive piggeries,
where more than 50% of sows showed high OD values

4.5 Discussion

In this survey, the prevalence of F4- and F18-specific antibodies in sera of 1,044 Cuban replacement gilts was assessed. By ELISA and diverse statistical analyses, a high seroprevalence of specific antibodies against the F4 and F18 fimbriae was confirmed.

Spread over the whole country, 26.8% and 5.6% of gilts were moderately and highly positives for F4-specific antibodies, respectively, and 33.6% were highly positives for F18-specific antibodies. Since a commercial vaccine against F18 is not available, and since vaccination of sows against $F4^+$ ETEC has not been practiced anymore during the last 15 years in Cuba (Wong *et al.,* 1995), our results show that $F4^+$ and $F18^+$ *E. coli* are highly prevalent as potential enteropathogens in Cuban piggeries. An $F4^+$ or $F18^+$ ETEC/VTEC infection provokes induction of F4- and F18-specific serum antibodies (Verdonck *et al.,* 2002).

Serological surveys using ELISA showed its reliability for testing vaccines and for epidemiological studies regarding fimbriae carrying *E. coli*. In Belgium, a seroprevalence study performed on sows showed that in West-Vlaanderen province, 79% of non-vaccinated farms were seropositive for F4-specific antibodies, while in Vlaams-Brabant only 31% were seropositive (Van den Broeck *et al.,* 1999b). The herd seroprevalence of F18-specific antibodies was higher in open system Belgian piggeries (96.4%) than in the closed system ones (88.8%; Verdonck *et al.,* 2003).

Specific diagnosis of swine colibacillosis is limited and out-of-date in Cuba. By using slide agglutination and direct immunofluorescence, Pedroso and Talavera (1983) detected $F4^+$ and $F5^+$ *E. coli* in fecal samples of diarrheic and healthy piglets in Havana province. In 1998, applying a whole-bacterial cell ELISA, $F4^+$ *E. coli* were identified in the 2.8% of diarrheic piglets from farms where sows were vaccinated against F4 and F5 in Camagüey province (Fuentes *et al.,* 2001). Lazo *et al.* (2005) reported 43.8% morbidity and 12.8% mortality due to enteric colibacillosis clinically diagnosed in Villa Clara province, Cuba.

After more than a decade, this is the first study that refers to a high prevalence of $F4^+$ *E. coli* in Cuba, specifically in the central region, whereas Blanco *et al.* (2006) did not find $F4^+$ *E. coli* in diarrheic piglets from the same region. To our opinion, they used a small sample size, and differences between their bacteriological (direct) and our immunological (indirect) identification are obvious. However, the high seroprevalence of F18-specific antibodies reported in our survey, with 33.6% serum samples showing high OD values, correlates with their report of 61% occurrence of F18 fimbriae among *E. coli* isolates.

Recent surveys carried out in China (Cheng *et al.,* 2006), USA (Zhang *et al.,* 2007), Zimbabwe (Madoroba *et al.,* 2009), Brazil (Vidotto *et al.,* 2009), and Vietnam (Oanh *et al.,* 2010) showed virulent *E. coli* carrying genes encoding F4 or F18 fimbriae as commonly associated pathogens with piglet's diarrhea or edema disease.

As colibacillosis is a multifactorial disease, our findings could not be directly associated with disease. However, in Poland, Osek (1999) found a higher prevalence of virulent *E. coli* in diarrheic weaned pigs than in healthy pigs, while $F4^+$ *E. coli* were only present in the diarrheics (19.1% prevalence). In China, Cheng *et al.* (2005) found a 58.33% occurrence of F18 fimbriae in *E. coli* strains isolated from pigs with diarrhea or edema disease.

After analyzing the high positivity to F4- and F18-specific antibodies obtained in our survey and based on previous studies that have been done in Belgium, a high genetic susceptibility of the Cuban swines to colonization by $F4^+$ and $F18^+$ *E. coli* could be inferred. By the in vitro adhesion assay, the 80% of Belgian pigs were classified in the F4 susceptible phenotype A ($F4acR^+$), and only 4% in the resistant phenotype E (Cox and Houvenaghel, 1987); it supports, in part, that in Flanders, 65% of non-vaccinated piggeries were seropositive for F4 (Van den Broeck *et al.,* 1999b). The 92% of Belgian pigs belonged to the FUT1M307G/G or FUT1M307G/A genotypes, both corresponding with sensitivity to $F18^+$ *E. coli* infections (Coddens *et al.,* 2007), which justified the high seroprevalence of F18-specific antibodies previously reported by Verdonck *et al.* (2003).

A low positive ELISA signal for F4- and F18-specific antibodies was obtained in 67.6% and 66.4% of the samples, respectively. Presumably, it could be the result of a weak bacterial infection determined by a good management and hygiene or by the prevalence of weak adhesion phenotypes. Yan *et al.* (2009) reported in China that the weak adhesion phenotypes of F4 (not yet described in Cuba) are present at a high frequency and mainly in crossbreds. As reported by Coddens *et al.* (2007) in F18 genetically susceptible piglets, weak and strong susceptible adhesion phenotypes were present in piglets aged from 1.5 to 23 weeks old.

The use of a vaccine against both F4 and F18 fimbrial adhesins could be one of many ways to control colibacillosis in Cuba. Tiels *et al.* (2008) recommended the FedF adhesin in combination with the F4 fimbriae as a good oral vaccine candidate, but more research on mucosal immunization is required.

In conclusion, this serological survey for F4- and F18-specific antibodies indicates that F4- and F18-fimbriated *E. coli* are widespread as potential etiologic agents of swine neonatal or post-weaning diarrhea and edema disease in Cuba. From the epidemiologic and zootechnic point of views, implementation of a national program for the prevention and treatment of swine colibacillosis appears advantageous.

4.6 Acknowledgments

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Chapter 5

Screening commercial pigs in Villa Clara province, Cuba, for mucin 4 *polymorphisms and susceptibility/resistance to F18*$^{+}$ E. coli

5.1 Abstract

F4 and F18 fimbriae frequently mediate the adhesion of pathogenic *E. coli* to specific receptors on the gut epithelium of pigs. The phenotypic expression of susceptibility or resistance to adhesion of F18 to the surface of enterocytes has been linked to *Cfo*I polymorphism in the *FUT1* gene (F18R). For F4, *Xba*I polymorphism in the porcine *mucin 4* gene has been linked with presence of the F4R, but not with absence. In order to know the frequency of genotypes and alleles determining susceptibility or resistance to F18 and *Xba*I polymorphism in the *mucin 4* gene in pigs raised in Villa Clara province, Cuba, genomic DNA of 90 F1 (Yorkshire x Landrace) x Duroc commercial pigs was tested by PCR-RFLP. Overall, the *Xba*I-resistant genotype was frequently detected (0.66), and the heterozygous or homozygous *Xba*I-digestible genotypes less frequently (0.31 and 0.03, respectively). These data compared with a previous survey reporting a high seroprevalence of F4-specific antibodies in sera of young sows in Cuba, confirm that *Xba*I polymorphism cannot be used as marker for F4R expression and supports the presence of other specific markers and or receptors. Pigs carrying the resistant genotype (AA) to $F18^{+}$ *E. coli* infections were not frequently found (0.13), and the susceptible (G) allele was highly frequent (0.73), coinciding with previous reports of a high prevalence of F18 fimbriae and antibodies against it in the Cuban swine herd.

5.2 Introduction

F4 and F18 fimbriae frequently mediate the adhesion of ETEC and F18 also of VTEC to specific receptors present on the surface of porcine enterocytes, leading to bacterial colonization and production of toxins, which provoke diarrhea (ETEC) or edema disease (VTEC; Fairbrother, 2006; Fairbrother and Gyles, 2006). Zhang *et al.* (2007) reported F4 and F18 as the major fimbrial antigens among *E. coli* isolates associated with diarrhea in young pigs in U.S.A.

DNA based-tests determining resistance or susceptibility of pigs to *E. coli* infections are important tools for breeding programs and for epidemiological studies undertaken to decrease the prevalence of swine colibacillosis in a herd. An *Xba*I polymorphism in intron 7 of the porcine *mucin 4* gene has been linked to the F4ab/ac fimbriae adhesive phenotype; by PCR-RFLP three genotypes of pigs regarding F4ab/ac mediated *E. coli* adhesion can be distinguished: resistant (RR), susceptible heterozygote (SR), and susceptible homozygote pigs (SS; Jørgensen *et al.,* 2004), however Rasschaert *et al*. (2007) demonstrated that the RR genotype also contained F4 susceptible pigs.

The F18R status of pigs is genetically determined (Bertschinger *et al.,* 1993), and susceptibility to *E. coli* colonization favored by F18 fimbriae appeared to be dependent on the activity of the *FUT1* gene, which encodes the alpha(1,2)-fucosyltransferase (Meijerink *et al.,* 1997; 2000). The discovery of the close linkage of a G→A polymorphism at nucleotide 307 on the *FUT1* gene and the F18R in porcine enterocytes has made the development possible of a PCR-RFLP using *Cfo*I enzyme digestion for identifying pigs resistant or susceptible to F18 adherence. Sequencing of the *FUT1* gene of pigs resistant (AA) to F18 revealed a transition G→A in both alleles on bp 307, resulting in an amino acid substitution at position 103 (Ala→Thr), different from the susceptible (GA or GG) pigs (Meijerink *et al.,* 1997).

Recently, a high seroprevalence of specific antibodies against F4 and F18 fimbriae was determined in the Cuban swine population (Chapter 4; de la Fé Rodríguez *et al.,* 2011). Furthermore, both fimbriae were most frequently identified on pathogenic *E. coli* isolated from suckling and weaned pigs with diarrhea in the Villa Clara province (Chapter 2). As a consequence, pigs raised in Cuba should have a high genetic susceptibility to these infections. Therefore we analyzed the receptor polymorphism for F18 fimbriae and determined *Xba*I polymorphism in the *mucin 4* gene of pigs raised in commercial farms located in the Villa Clara province.

5.3 Materials and methods

Survey design and collection of blood samples

Blood was collected from the orbital venous sinus of 90 commercial pigs F1 (Yorkshire x Landrace) x Duroc equally distributed (15 per piggery) in six large continuously farrowing piggeries (namely

from A to F) located in Villa Clara province, Cuba. One ml of blood was gently mixed with 100 µl of 10% EDTA to prevent coagulation and was kept at -20°C after arrival to the laboratory. These pigs were also surveyed in Chapter 2 of this thesis.

Extraction of genomic DNA

Red blood cells were lysed by mixing 100 µl of blood with 1 ml of TE buffer (10 mM Tris-HCL + 1 mM EDTA, pH 7.5), this mixture was centrifuged at 3800x*g* for 20 sec, and the pellet was washed twice under the same conditions. Next, 2 µl of proteinase K (20 mg/ml, Invitrogen) diluted in 200 µl of K-buffer (50 mM KCL + 20 mM Tris-HCL + 2.5 mM $MgCl_2$ + 0.5% Tween®20, pH 8.3) were added to the pellet, and incubation was performed at 56°C for 1 h in a warm water bath. Next, proteinase K was inactivated by incubation at 95°C for 10 min, centrifugation was performed at 3800x*g* for 20 sec, and the obtained supernatant was stored at -20°C, and used as template DNA for PCR analyses.

DNA test for mucin 4 *polymorphism*

PCR-RFLP was performed as described by Jørgensen *et al.* (2004). Briefly, the PCR reaction (20 µl) which allows the amplification of the *mucin 4* gene fragment contained 5 pmol of each primer (5′-GTGCCTTGGGTGAGAGGTTA-3′/5′-CACTCTGCCGTTCTCTTTCC-3′), 200 µM of each dNTPs (Roche Diagnostics GmbH), 2 mM of $MgCl_2$ (Promega), 0.5 U of SuperTaq (HT Biotechnology Ltd.), 2 µl of 10xSuperTaq buffer, and 5 µl of template DNA. The PCR amplifications were performed in a thermal cycler PTC-100™ (MJ Research Inc.) by incubation of the mixture at 95°C for 5 min for pre-denaturation, followed by 39 cycles of denaturation at 95°C for 15 sec, annealing at 65°C for 30 sec, and extension at 72°C for 1 min, with a final extension at 72°C for 7 min. Finally, 5.5 µl of the PCR product (367 bp) were digested with *Xba*I (Promega) for 2 h at 37°C in warm water bath. One allele (R) is resistant to *Xba*I digestion, whereas the S allele is digested into a 151 bp and a 216 bp fragment.

DNA marker-based test for the F18R

The PCR reaction (30 µl) for the amplification of the *FUT1* gene fragment contained 15 pmol of each primer (5′-CTTCCTGAACGTCTATCAAGACC-3′/5′-CTTCAGCCAGGGCTCCTTTAAG-3′), 200 µM of each dNTPs (Roche Diagnostics GmbH), 1.5 mM of $MgCl_2$ (Promega), 0.5 U of SuperTaq (HT Biotechnology Ltd.), 3 µl of 10xSuperTaq buffer, and 5 µl of template DNA. The PCR amplifications were performed by incubation of the mixture at 95°C for 3 min for pre-denaturation, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 61°C for 30 sec, and extension at 72°C for 30 sec, the final extension was at 72°C for 10 min. In order to detect the G→A polymorphism at

nucleotide 307 of the *FUT1* gene described by Meijerink *et al.* (1997), ten µl of the PCR product (421 bp) were digested with *Cfo*I (Promega) for 2 h at 37°C in a warm water bath, resulting in fragments of 328 and 93 bp for the resistant (A) allele, and fragments of 241, 93, and 87 bp for the susceptible (G) allele.

In every piggery and in the overall sample the genotypes frequency was determined dividing the number of every genotype by the number of tested pigs; the alleles frequency was determined dividing the number of every allele by the total number of alleles in every group.

5.4 Results and discussion

The *mucin 4* and *FUT1* genes fragments were successfully amplified in all pigs, and the digestion with *Xba*I and *Cfo*I enzymes, respectively, were reliable for detecting polymorphisms in both of them, allowing the detection of susceptible and resistant pig genotypes to *E. coli* colonization mediated by F4 or F18 fimbriae (Fig. 1, 2, and 3).

Mucin 4 *gene polymorphisms*

As shown in Table 1, pigs homozygous for indigestible alleles (RR) are highly frequent (0.66), Jørgensen *et al.* (2004 associated this status to resistance to *E. coli* adhesion mediated by F4ab/ac fimbriae. However, in our previous study we determined a high seroprevalence of F4-specific antibodies in gilts from Cuban piggeries (Chapter 4; de la Fé Rodríguez *et al.,* 2011). These findings support findings of Rasschaert *et al*. (2007) that the F4R should not only be linked to the activity of the *mucin 4* gene. Indeed, Rasschaert *et al.* (2007) found that F4ab/ac fimbriae could adhere to villous brush borders of pigs that carry only *Xba*I-indigestible alleles (RR), and reported the absence of the *Xba*I restriction site in the *mucin 4* gene fragment of 33.3% pigs positive for the adhesion of *E. coli* expressing F4ab/ac fimbriae. Although Erickson *et al.* (1992; 1994) proposed the intestinal mucin-like sialoglycoproteins (IMTGPs) as F4R, also other molecules have been proposed as receptors such as an intestinal transferrin (GP74; Grange and Mouricout, 1996), an intestinal glycosphingolipid (Grange *et al*., 1999) and the porcine aminopeptidase-N (Rasschaert*,* 2008) have been proposed as F4R.

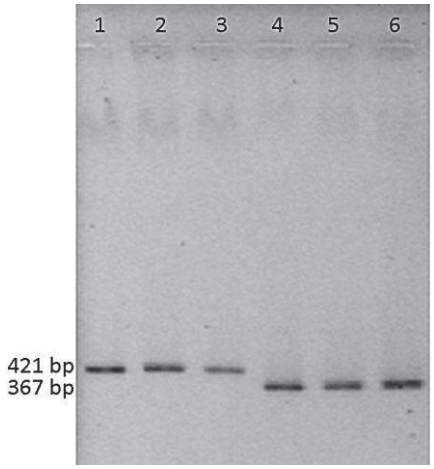

Figure 1. Agarose gel electrophoresis showing examples of the PCR product obtained from the amplification of the *FUT1* (421 bp; samples 1, 2, and 3) and *mucin 4* (367 bp; samples 4, 5, and 6) gene fragments.

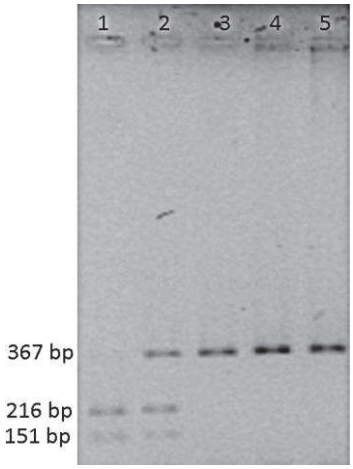

Figure 2. Agarose gel showing examples of the product obtained after *Xba*I digestion of the *mucin 4* gene fragment. Pig 1 is homozygous for digestible alleles (SS). Pigs 3, 4, and 5 carry indigestible alleles (RR), and pig 2 is heterozygous (SR).

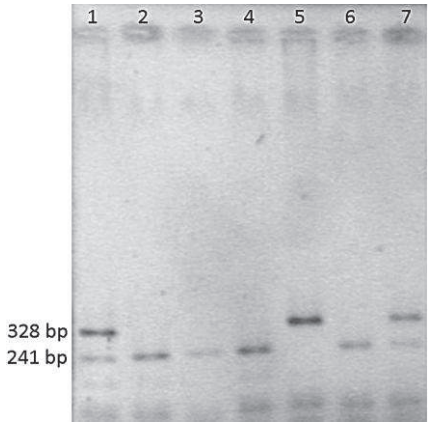

Figure 3. Agarose gel electrophoresis showing examples of the product obtained after *Cfo*I digestion of the amplified *FUT1* gene fragment. Pigs 1 and 7 carry the GA susceptible genotype. Pigs 2, 3, 4, and 6 carry the GG susceptible genotype, and pig 5 carry the AA resistant genotype.

Table 1. The frequency of pigs carrying homozygous *mucin 4* gene for the presence of *Xba*I restriction site (SS), homozygous for its absence (RR), and heterozygous (SR) in the Villa Clara province, Cuba.

Piggery	**Genotypes frequency of *Xba*I digestion patterns of the *mucin 4* gene**			**Alleles frequency**	
				*Xba*I-indigestible	*Xba*I-digestible
	RR	SR	SS	R	S
A	0.80	0.20	0	0.90	0.10
B	0.60	0.40	0	0.80	0.20
C	0.93	0.07	0	0.97	0.03
D	0.40	0.53	0.07	0.67	0.33
E	0.73	0.20	0.07	0.83	0.17
F	0.47	0.47	0.07	0.70	0.30
Total	0.66	0.31	0.03	0.81	0.19

FUT1 *gene polymorphisms and F18R*

Pigs carrying the resistant genotype (AA) to F18 were not frequently found (0.13; Table 2). Similar results were obtained by Shi *et al.* (2002) in 158 pigs of Yorkshire, Landrace, and Duroc breeds in China, where the frequency of the resistant genotype AA was 0.11 and the frequency of the susceptible genotypes GA and GG were 0.35 and 0.54, respectively. In 74 Belgian Landrace x Pietrain or Dutch Landrace an even lower frequency of the resistant genotype AA (0.08) was detected and the frequency of the susceptible genotypes GA and GG were 0.37 and 0.55, respectively (Coddens *et al.,* 2007).

Table 2. The frequency of F18 susceptible (GG, GA) and resistant (AA) genotypes identified by the *Cfo*I polymorphism in the *FUT1* gene of 90 pigs in the Villa Clara province, Cuba.

Piggery	**Genotypes frequency**			**Alleles frequency**	
	AA	GA	GG	A	G
A	0	0.40	0.60	0.20	0.80
B	0.07	0.27	0.67	0.20	0.80
C	0.27	0.20	0.53	0.37	0.63
D	0.13	0.13	0.73	0.20	0.80
E	0.13	0.13	0.73	0.20	0.80
F	0.20	0.47	0.33	0.43	0.57
Total	0.13	0.27	0.60	0.27	0.73

The high genetic susceptibility for adhesion of F18$^+$ *E. coli* in Cuban pigs in our study is in agreement with the high prevalence of F18-specific antibodies in young sows we reported previously (Chapter 4; de la Fé Rodríguez *et al.,* 2011) and with the high occurrence (61%) of the F18 coding gene in *E. coli* isolated from piglets with diarrhea in Central Cuba in a study of Blanco *et al.* (2006) and in ETEC and VTEC strains reported in Chapters 2 and 3 of this thesis.

The presence of the allele A determining F18 resistance at the frequency of 0.27 in commercial pigs is important for future breeding programs aiming the selection of F18-resistant breeding stocks in order to prevent diarrhea or edema disease in Cuba.

It is interesting that Chinese native pig breeds (Taihu, Huai) lacking genetic factors providing resistance to F18 fimbriae, have stronger resistance to post-weaning diarrhea and edema disease compared with western pig breeds due to a reduced growth rate of the Chinese pigs (Bao *et al.,* 2008).

At least for what F18$^+$ *E. coli* control is concerned, positive selection of Cuban pigs for the AA F18-resistant genotype could contribute to decrease swine diarrhea and prevent edema disease.

5.5 Acknowledgments

We thank especially Dr. Annelies Coddens and Ir. Phillip Bellot from the Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, for their assistance in detecting polymorphisms in the *mucin 4* and *FUT1* genes. This study was carried out in the frame of an IUC program between VLIR-UOS and Universidad Central “Marta Abreu” de Las Villas.

Chapter 6

General discussion and future perspectives

6.1 General discussion

Cuban swine veterinary practitioners often complain about the high occurrence and yearly increased incidence of diarrhea in young pigs, as well as about the limitations for the specific identification of its etiology (e.g. enteropathogens or lack of food/water quality), which results in the application of insufficient prevention/control measures at national level like the common uncontrolled administration of antibiotics (Cabrera *et al*., 2010). Diarrhea diagnosis in Cuban piggeries and provincial Veterinary Diagnostic Laboratories is frequently restricted to clinical and macropathological examinations (Cabrera and García, 2009), which are a tentative rather than a specific diagnostic tool (Elicker *et al*., 2010). Indeed, the clinical picture of porcine neonatal or post-weaning diarrhea often do not point to a likely etiology because of the multifactorial nature of this disease and the variation in clinical signs and epidemiology that can be produced by the effect of one or more disease agents (Straw *et al*., 2006). In 2008, the Cuban Institute for Swine Research reported gastroenteric diseases to cause 31% and 37% of the total piglet's mortality during the pre-weaning and post-weaning periods, respectively. Also, other causes of piglet's mortality related with diarrhea, like crushing or hypoglycemia, frequently occur in Cuban piggeries (Cabrera and García, 2009). Moreover, research, technology transfer, and laboratory protocols contributing to the epidemiological characterization of swine enteric infections have been limited during the last two decades and are out of date in Cuba (Pedroso and Talavera, 1983; Cabrera and García, 1985; Koudela *et al*., 1989; NRAG, 1989; Fuentes *et al*., 2001; Barrera *et al*., 2005; Blanco *et al*., 2006).

The epidemiology of any infectious syndrome is a wide matter to deal with, specifically when previous information is almost completely lacking. We had thus first to identify the pathogens currently associated with piglet's diarrhea in the Villa Clara province of Cuba. Then, pathogenic *E. coli,* which were the most frequent enteropathogen, were characterized for virulence factors, antibiotic susceptibility/resistance, and genetic profiles. Later, a seroprevalence study of antibodies against F4 and F18 fimbriae all over Cuba contributed to the epidemiological picture of *E. coli* commonly associated with piglet's diarrhea. Finally, the enteric receptor status for F18 fimbriae and the *Xba*I polymorphisms in the porcine *mucin 4* gene linked with presence, but not with absence of the F4R, of commercial pigs in Cuba were tested by DNA based-markers.

Failure of isolation and identification of certain enteropathogens actively involved in the pathogenesis of diarrhea in a swine herd could lead to the failure of a control program. Diseases such as TGE, coccidiosis, rotavirosis, colibacillosis and clostridial enteritis are difficult to eliminate once they have become a problem in the herd; and they may still contribute to diarrhea due to failures in preventive programs (antibiotic resistance, vaccinations, sanitation, or management; Straw *et al*., 2006). We observed that 64.4% and 42.2% of suckling piglets and weaned pigs with diarrhea, respectively, were infected with at least one of the following enteropathogens: ETEC, VTEC, α-

toxigenic *C. perfringens, S.* Newport, TGEV, rotavirus A, *C. parvum* or *I. suis*, and their frequency ranged from 3.3% for VTEC to 25.6% for ETEC. Important enteropathogens like β-toxigenic *C. perfringens,* PEDV, *Eimeria* spp., and helminths were not detected, but we cannot confirm their absence in Cuba or even in Villa Clara as limited numbers of pigs during a limited period of time were surveyed. Swine helminths are common in Cuba as a tropical country: *Hyostrongylus rubidus*, *Strongyloides* sp., *Ascaris suum, Macracanthorhynchus hirudinaceus, Oesophagostomum* sp., *Trichuris suis*, and even *Fasciola hepatica* have recently been reported in pigs raised outdoors by private farmers in the Villa Clara province (de la Fé Rodríguez *et al.,* 2007).

Eight kinds of combined infections were identified in 25% of enteropathogen positive pigs. To our point of view, mixed infections have to be accurately identified to plan and implement an efficient disease control. Worldwide few controlled experiments have been designed to study the interaction between swine enteropathogens (Chapter 1). Lecce *et al*. (1982) and Enemark *et al. (*2003a) reported that a mixed infection rotavirus-ETEC or rotavirus-*Cryptosporidium*, respectively, causes a dramatic aggravation of diarrhea and clinical signs while mild clinical signs of illness were observed in piglets mono-infected with rotavirus, ETEC or *Cryptosporidium*. In Japan, Ushida *et al*. (2009) closely associated mixed infections with diarrhea as 42% of diarrheic weaned pigs had multi-infections, whereas such infections only occurred in 17% of weaned pigs with solid feces, and 33 % of diarrheic suckling piglets showed mixed infections, whereas they were absent in piglets without scour.

In this study we found that pathogenic *E. coli* were commonly associated with porcine pre- or post-weaning diarrhea. Seventeen clones of pathogenic *E. coli* isolated from diarrheic piglets carried genes for virulence factors F4, F5, F6, F18, F41, STa, STb, STx2e, or LT. $F4^+$ and $F18^+$ ETEC were the most frequently identified and they showed multi-resistance to most of antibiotics routinely administered in Cuban piggeries (i.e. tetracycline, ampicillin, sulphonamides, and kanamycin) in contrast to the $F18^+$/$STx2e^+$ isolates (clonal group XI) which were only resistant to sulphonamides. These are important findings to manage in Cuban piggeries because of the fact that these pathogenic bacteria were isolated from diarrheic pigs, and they have been associated with clinical disease worldwide: in Poland, Osek (1999) isolated $F4^+$ *E. coli* only in diarrheic weaned pigs (19.1% prevalence), and in China, Cheng *et al*. (2005) reported a 58.33% occurrence of $F18^+$ *E. coli* in pigs suffering from diarrhea or edema disease. Boerlin *et al*. (2005) and Travis *et al*. (2006) reported linkages of antibiotic resistance and virulence genes on plasmids in porcine ETEC; consequently, uncontrolled administration of antibiotics, like is happening in Cuban piggeries, may contribute to the selection, persistence, and spreading of pathogenic *E. coli*. Therefore, the fact that ETEC showed susceptibility to several antibiotics less or never administered in Cuban piggeries (e.g. nalidixic acid, ciprofloxacin, gentamicin, trimethoprim, or cefazolin) has to be interpreted and implemented with caution. From our point of view, administration of new antibiotics in Cuban piggeries currently, will

only attenuate temporally the effects of pathogenic enterobacteria, and will favour the selection of resistant clones. In Cuban piggeries, improvements on the management of pregnant sows, a good attention to the parturition, sufficient intake of colostrum by newborn piglets, vaccination of young pigs and sows against enteropathogens, as well as improvements on housing and biosecurity will decrease diarrhea occurrence, and will decrease the demand of antibiotics.

In 29% and 38% of piggeries, 50% or more sows showed moderate or high levels of serum F4- and F18-specific antibodies, respectively. As vaccination against F4 has not been practiced for the last 15 years in Cuba (Wong *et al*., 1995), and an $F4^+$ or $F18^+$ ETEC/VTEC infection induces F4- or F18-specific antibodies (Verdonck *et al.,* 2002), we can confirm that the $F4^+$ and $F18^+$ *E. coli* are highly prevalent as potential enteropathogens in Cuban piggeries. This is in contradiction with Blanco *et al.* (2006) who could not isolate $F4^+$ *E. coli* in Central Cuba; in addition, we reported multidrug-resistant $F4^+$ ETEC in the central province Villa Clara.

The high seroprevalence of F4- and F18-specific antibodies in sera of young sows in Cuba (Chapter 4) infer a high genetic susceptibility of pigs raised in Cuban piggeries for colonization by $F4^+$ and $F18^+$ *E. coli*.

Susceptibility to colonization by $F18^+$ *E. coli* is dependent on the glycosylation status influenced by the activity of the *FUT1* gene, which encodes the alpha(1,2)-fucosyltransferase (Meijerink *et al.,* 1997; 2000). Similar to reports by Shi *et al*. (2002) in Yorkshire, Landrace, and Duroc breeds in China and by Coddens *et al*. (2007) in Belgium, pigs carrying the resistant genotype (AA) were not frequently found (13%; Chapter 5). This finding is in agreement with the high prevalence of F18-specific antibodies previously reported, and with the high occurrence (61%) of the F18 fimbrial adhesin among pathogenic *E. coli* isolated from diarrheic piglets in Central Cuba (Blanco *et al*., 2006). A genetic improvement of pigs raised in Cuba considering the AA resistant genotype to F18 mediated adhesion could be taken as a way to control swine diarrhea or edema disease.

The high seroprevalence of F4-specific antibodies, even though the resistant genotype described by Jørgensen *et al*. (2004) was highly present (66%) in commercial F1 (Yorkshire x Landrace) x Duroc pigs raised in Cuba supports that the polymorphism in *mucin 4* is not a reliable marker for F4ab/ac receptor expression as reported by Rasschaert *et al*. (2007) or that other receptors are involved. Indeed, other F4R candidates have been reported such as an intestinal transferrin (GP74; Grange and Mouricout, 1996), an intestinal glycosphingolipid (Grange *et al*., 1999), and the porcine aminopeptidase-N (Rasschaert, 2008).

Diarrhea is a multifactorial disease influenced by management and environmental conditions, the interaction with the causative agents and host factors like immunity and genetic susceptibility

(Fairbrother, 2006; Fairbrother and Gyles, 2006). Apart from the stressful weaning, also housing and management aspects could play a role in the appearance of diarrhea outbreaks in Cuban piggeries:

- Big swine farms (> 500 sows) with an open system and all pig categories included are common, making health control difficult.
- A mixture of management failures such as administration of a not well-balanced ration to pregnant and lactating sows which affect their body condition, the presence of sows with an excess number of parturitions and incorrect care of parturition lead to a mixture of problems such as low weight at birth, and also low survival rate of newborn piglets due to mastitis-metritis-agalactia syndrome and or low milk production in sows (Lazo and Gutiérrez, 2011), insufficient intake of colostrum, loss of blood through the umbilical cord, and hypoglycemia.
- Open stables expose pigs to thermal stress by the combination of high environmental temperature and humidity. The lower survival rate (69.7%) of suckling piglets during 2008 was in September and October when, as usual, Cuba was affected by two potent hurricanes. Due to this tropical climate, power supply, water quality, feeding, housing, and sanitation are negatively impacted (Cabrera *et al*., 2010).
- High density of insect vectors like flies inside installations. Recent surveys performed in Taiwan (Wang *et al*., 2011) and the Czech Republic (Literak *et al*., 2009) have implicated flies as vectors for transmission of antibiotic resistant enterobacteria in swine farms.
- Lack of good pre-starting feed (Cabrera *et al*., 2010) and good feeding management before and after weaning.
- Lack of administration and inadequate control of the herd book.
- Another aspects directly attempt against biosecurity such as low quality of sanitation and disinfection (Cabrera et al., 2010) and the fact that stables are open which make hygiene control difficult. Also, sometimes there is no delimitation of the ¨dirty¨ and ¨clean¨ areas inside the piggery and breeding of pigs by the personal of the piggery at their home is common which increases the risk of introduction of pathogens to the piggery, mostly when is not ruled the change of boots and clothing before entering the ¨clean¨ area. The common interchange of breeding stock pigs among farms and the housing of sows in large groups could also contribute to the spreading of enteropathogens.

6.2 Future perspectives

Most of the recent epidemiological data related to swine diarrhea in Cuba do not reach a casual diagnosis or are from studies done in the frame of scientific projects of educational institutions. In order to improve swine health in Cuba, the country has to reinforce the Regional Veterinary Diagnostic Laboratories, which are responsible for the analysis of diarrhea outbreaks, with an improvement of their infrastructure based on the experience and research results of Universities and other Institutions not directly linked with the National Institute of Veterinary Medicine like the National Centre for Animal Health and the Cuban Institute for Swine Research. The improvement of enteropathogen diagnosis at national level, as well as the implementation of efficient prevention and control programs for piglet diarrhea, particularly due to ETEC, might have a positive impact on the performance of young pigs in Cuba.

Due to the application of general laboratory protocols, current reports related with swine diarrhea in Cuba are not specific enough (Ricardo, 2008; Cabrera and Garcia, 2009; Cabrera *et al*., 2010) so that non-infectious processes like alimentary diarrhea (e.g. non-infectious hyper-lactate or dyspeptic diarrhea; Ushida *et al.*, 2009) mask infectious diarrhea and vice versa. Specifically, we recommend to correctly implement the guidance NRAG (1989) of the Cuban Ministry of Agriculture which rules veterinary diagnostics, microbiological classification and assay procedures in the Veterinary Diagnostic Laboratories. Furthermore, laboratory protocols for the identification of enteropathogens need to be updated. For example, it might be favorable to include virotyping of *E. coli* and toxinotyping of *C. perfringens* by multiplex-PCR during epidemiological analysis and control of swine diarrhea in Cuba.

Pathogenic *E. coli* associated with diarrhea in young pigs appeared resistant to most antibiotics routinely employed in Cuban piggeries. We can infer that other pathogenic bacteria associated with diarrhea or other diseases, and even in other domestic animals or humans, are also resistant to antibiotics currently applied in Cuba. This can result in severe health risks due to outbreaks of multiresistant pathogens. We strongly recommend that Cuban veterinary authorities establish adequate antibiotic resistance surveillance, which is absent, all over the country. The logistic and administrative instances from the National Institute of Veterinary Medicine should consider that instead of cheap and indiscriminately employed antibiotics like tetracycline, a well managed antibiotic strategy together with good management practices of pigs can help to reduce antimicrobial resistance and disease in Cuban piggeries.

The reduction of the morbidity in young pigs due to colibacillosis from 8.3% in 1993 to 0.9% in 1995 in the Camagüey province, Cuba, coincided with the application of the national recombinant vaccine Vacoli, which contained the antigens K88 and K99 (Wong *et al*., 1995). Nowadays, it might be necessary to re-introduce vaccination against pathogenic *E. coli* in Cuban piggeries. Results of this

thesis suggest that F4 and F18 fimbriae as well as enterotoxins are good candidates to be considered for this preventive strategy. Tiels *et al*. (2008) recommended FaeG adhesin (F4) in combination with FedF adhesin (F18) as a good oral vaccine candidate. Recently, Ruan *et al*. (2011) demonstrated that the fusion antigen FaeG-FedF-LT(192)A2:B elicits antibodies that neutralize LT toxin and inhibit the adherence of F4 and F18 fimbriated *E. coli*.

We think that the presence of the allele A determining F18 resistance at the frequency of 27% in Cuban swines could contribute to future breeding programs aimed at selection of F18-resistant breeding stock pigs in order to prevent diarrhea or edema disease. Selection and breeding of F4-resistant pigs also appear advantageous.

In conclusion, results of this thesis indicate that the economic performance of the Cuban swine industry might be favored by intervention strategies undertaken to improve enteropathogens' diagnosis and to better manage antibiotic use. Prevention programs of diarrhea should be reinforced by vaccination against *E. coli* and other enteropathogens, by breeding pigs resistant to $F18^+$ *E. coli*, and by improving housing and management in piggeries.

Summary

Porcine pre- and post-weaning diarrhea are multi-factorial diseases that negatively impact the efficiency of swine production worldwide. Often, swine veterinarians complain about the scarce information on the epidemiology of diarrhea and about limitations for the specific identification of enteropathogens in Cuba. Therefore, insufficient control measures are undertaken which results in an increased incidence of swine diarrhea.

This thesis updates epidemiological knowledge concerning the infectious etiology of diarrhea in young pigs in Cuba through the differential identification of enteropathogens in neonatal, in suckling and in newly-weaned piglets. This epidemiological study identified *Escherichia coli* as a major enteropathogen. Therefore, a major focus was directed on *E. coli* infections in the rest of the thesis. First, the antibiotic resistance and the genetic relatedness of Cuban pathogenic *E. coli* isolates were determined. Then, the seroprevalence of antibodies against F4 and F18 fimbriae of *E. coli* was assessed. Finally, the genetic susceptibility of Cuban pigs for enteric colonization by $F4^+$ and $F18^+$ *E. coli* was analyzed.

The **first Chapter** is a review of literature focused on the swine production in the Cuban context and on the epidemiology of the main enteropathogens causing diarrhea in young pigs worldwide. The identification of different enteropathogens is also discussed and the epidemiological term ¨mixed types¨ which refers to the combined infectious etiology of diarrhea is introduced.

Chapter 2 was undertaken to gain insights into the infectious etiology of porcine pre- and post-weaning diarrhea in Villa Clara province, Cuba. Intestinal contents of diarrheic pigs were tested by culture, microscopy, and immunological or DNA-based techniques. At least one enteropathogen was detected in 64.4% and in 42.2% of suckling and weaned pigs, respectively. Enterotoxigenic *E. coli* (ETEC) was significantly the most frequent pathogen, and most virotypes were either STa^+/STb^+ or $F4^+/STa^+/STb^+$. The overall occurrence of the rest of the pathogens was 10% for transmissible gastroenteritis virus and *Cryptosporidium parvum*, 6.7% for rotavirus A and *Isospora suis*, 5.6% for α-toxigenic *Clostridium perfringens*, 3.3% for verocytotoxigenic *E. coli* (VTEC), and 2.2% for *Salmonella enterica* subspecies *enterica* serotype Newport. Porcine epidemic diarrhea virus, β-toxigenic *C. perfringens, Eimeria* spp., and helminths were not identified. Twelve out of 48 enteropathogen positive piglets (25%) were infected with more than one pathogen and ETEC was present in 10 out of 12 mixed infections. These results demonstrate that several enteropathogens, either alone or as part

of a mixed infection, are associated with porcine pre- and post-weaning diarrhea in the Villa Clara province, Cuba.

Since *E. coli* is the most prevalent enteropathogen in pigs suffering from diarrhea in Cuba, we studied this pathogen in more detail in the next Chapters.

In **Chapter 3** we determined the antibiotic resistance profile and the genetic relatedness of pathogenic *E. coli* isolated from piglets suffering from diarrhea. The highest resistance rates were seen to antibiotics traditionally administered in Cuban piggeries: tetracycline (69%), ampicillin (54%), sulphonamide compounds (50%), and kanamycin (50%); 65% of isolates were multi-drug resistant. The ERIC-PCR revealed a high degree of polymorphism in the *E. coli* DNA sequences and relatedness among $F4^+/STa^+/STb^+$ or $F18^+/LT^+/STb^+$ isolates from different piggeries, and among the STb^+, STa^+/STb^+, $F4^+/STa^+/STb^+$ or $F18^+/STx2e^+$ ones from the same piggery. Genetically diverse as well as genetically related pathogenic *E. coli* highly susceptible to nalidixic acid, ciprofloxacin, gentamicin, amikacin, chloramphenicol, cephalosporins, amoxicilline-clavulanic acid, and trimethoprim are associated with piglet's diarrhea in the Villa Clara province, Cuba. The analysis on genetic diversity by ERIC-PCR demonstrated a clonal relationship among pathogenic *E. coli* carrying the same virulence factors and similar antibiotic resistance. The implementation of these epidemiological research data throughout Cuba can contribute to the surveillance, prevention, and control of swine colibacillosis.

Considering that F4- or F18-specific serum antibodies in Cuban pigs will reflect the presence and spread of $F4^+$ ETEC or $F18^+$ ETEC/VTEC, we determined in **Chapter 4** the prevalence of F4- and F18-specific antibodies in sera of 1044 gilts. For the data analysis random-effects models and a mixture model in R (package "mixAK"; Komárek, 2009) were fitted. Low, moderate, and high levels of F4-specific antibodies were found in 67.6%, 26.8%, and 5.6% of gilts, while 66.4% and 33.6% of them showed low and high levels of F18-specific antibodies, respectively. Hereby, we show that $F4^+$ and $F18^+$ *E. coli* are highly prevalent as potential enteropathogens in Cuban piggeries.

Chapter 5 was carried out to firstly know the frequency of genotypes and alleles determining susceptibility or resistance to $F18^+$ *E coli* infections. To investigate the susceptibility to $F4^+$ *E. coli* infections, the *Xba*I polymorphism in the porcine *mucin 4* gene was determined in pigs raised in Villa Clara province, Cuba. The *Xba*I-resistant genotype was frequently detected (0.66) and the susceptible heterozygote or homozygote genotypes were less frequent (0.31 and 0.03, respectively). Since we found that there is a high seroprevalence of F4-specific antibodies in sera of young sows in Cuba, we conclude that the *XbaI* polymorphism in *mucin 4* cannot be used as a reliable marker to determine genetic resistance to $F4^+$ *E. coli* infections.

Pigs carrying the F18-resistant genotype were not frequently found (0.13), while the heterozygous or homozygous susceptible genotypes occurred most frequently (0.27 and 0.60, respectively), coinciding with previous reports of a high prevalence of $F18^+$ *E. coli* and F18-specific antibodies in the Cuban swine herd. At least for $F18^+$ *E. coli* control, a genetic improvement of pigs considering the resistant genotype could be taken as a way to control swine diarrhea or edematous disease in Cuba. This strategy is not recommended for F4 since the *Xba*I polymorphism in the *mucin 4* gene does not appear to be a good marker for resistance to $F4^+$ *E. coli* infections.

Finally, the general discussion and future perspectives are presented in **Chapter 6**. A better production efficiency of the Cuban Company for pork production might be achieved by the improvement of surveillance, prevention, and control programs of infectious diarrhea in young pigs, particularly colibacillosis. Cuban veterinary authorities should acknowledge the importance of identification of enteropathogens and of surveillance of antibiotic resistance by the Veterinary Diagnostic Laboratories. In the short-term, vaccination against *E. coli*, the introduction of efficient antibiotics and improvement of management practices appear advantageous to control porcine pre- and post-weaning diarrhea in Cuba. In the long-term, breeding of F4- and F18-resistant pigs as well as investments in facilities will be necessary.

Samenvatting

Diarree bij neonatale en pasgespeende biggen is een multifactoriële ziekte die economische schade berokkent aan varkenshouders wereldwijd. Voor Cubaanse dierenartsen is er weinig informatie beschikbaar over de epidemiologie van diarree bij jonge biggen en is de kennis over de identiteit van de enteropathogenen gelimiteerd. Daardoor kunnen onvoldoende controle maatregelen genomen worden wat resulteert in een verhoogd voorkomen van varkensdiarree.

Deze doctoraatsthesis geeft recente inzichten in de etiologie van diarree bij jonge biggen in Cuba. Hiertoe werd identificatie van de verschillende enteropathogenen aanwezig in faeces van biggen met diarree uitgevoerd. Omdat *Escherichia coli* de meest voorkomende pathogeen bleek, werd van deze *E. coli* stammen ook het antibioticumresistentieprofiel en de genetische verwantschap bepaald. Bovendien werd de seroprevalentie van specifieke antistoffen tegen de fimbriae van $F4^+$ *E. coli* en $F18^+$ *E. coli* -die gekend zijn als de meest voorkomende *E. coli* betrokken bij varkensdiarree- onderzocht en werd de genetische gevoeligheid van biggen voor $F4^+$ en $F18^+$ *E. coli* infecties bepaald.

In het **eerste Hoofdstuk** wordt een overzicht gegeven van recente literatuur omtrent de varkensproductie in Cuba en omtrent de epidemiologie van de belangrijkste enteropathogenen die diarree veroorzaken bij jonge biggen wereldwijd. Hier wordt ook de identificatie van de verschillende enteropathogenen besproken en wordt de epidemiologische term 'Mixed types' geïntroduceerd, die verwijst naar de aanwezigheid van verscheidene pathogenen in faeces van biggen die door diarree getroffen zijn.

Het onderzoek in **Hoofdstuk 2** werd ondernomen om inzicht te verwerven in de infectieuze oorzaken van diarree bij neonatale en pasgespeende varkens in de Villa Clara provincie in Cuba. Darminhouden van biggen met diarree werden getest door cultivatie, microscopie en immunologische of DNA-gebaseerde technieken. Ten minste 1 enteropathogeen werd gedetecteerd in respectievelijk 64.4% en 42.2% van de zuigende en gespeende biggen. Enterotoxigene *E. coli* (ETEC) was significant de meest voorkomende pathogeen en de meeste virotypes waren ofwel STa^+/STb^+ of $F4^+/STa^+/STb^+$. Het algemeen voorkomen van de overige geteste pathogenen was 10% voor transmissibel gastroenteritis virus en *Cryptosporidium parvum*, 6.7% voor rotavirus A en *Isospora suis*, 5.6% voor α-toxigene *Clostridium perfringens*, 3.3% voor verotoxigene *E. coli* (VTEC) en 2.2% voor *Salmonella enterica* subspecies *enterica* serovar Newport. Het porciene epizoötische diarree virus, β-toxigene *C. perfringens*, *Eimeria* spp. en helminthen werden niet teruggevonden. Twaalf van de 48 enteropathogeen-positieve varkens (25%) werden geïnfecteerd met meer dan één

pathogeen en ETEC was aanwezig bij 10 van de 12 gemengde infecties. Deze resultaten tonen aan dat verschillende pathogenen, ofwel individueel ofwel als onderdeel van een gemengde infectie, geassocieerd kunnen worden met varkensdiarree in neonatale en pasgespeende biggen in de Villa Clara provincie in Cuba.

Gezien *E. coli* de meest voorkomende enteropathogeen is bij biggen met diarree in Cuba, werd deze pathogeen meer gedetailleerd bestudeerd in de volgende hoofdstukken.

In **Hoofdstuk 3** werd het antibioticumresistentieprofiel en de genetische verwantschap tussen pathogene *E. coli* isolaten van biggen die lijden aan diarree onderzocht. De hoogste graad van antibioticumresistentie werd gevonden bij antibiotica die traditioneel toegediend worden bij Cubaanse varkensbedrijven, namelijk tetracycline (69%), ampicilline (54%), sulfonamides (50%) en kanamycine (50%). Vijfenzestig procent van de isolaten waren resistent tegen meerdere antibiotica. De ERIC-PCR toonde aan dat er een hoge graad van polymorfismen is in de *E. coli* DNA sequenties en dat er een hoge graad van verwantschap is tussen $F4^+/STa^+/STb^+$ of $F18^+/LT^+/STb^+$ isolaten van verschillende varkenshouderijen en tussen de STb^+, STa^+/STb^+, $F4^+/STa^+/STb^+$ of $F18^+/STx2e^+$ isolaten. Zowel genetisch diverse als genetisch verwante pathogene *E. coli* die sterk gevoelig zijn voor nalidixinezuur, ciprofloxacine, gentamycine, amikacine, chloramphenicol, cephalosporines, amoxicilline-clavulaanzuur en trimethoprim zijn geassocieerd met diarree van biggen in de Villa Clara provincie in Cuba. De analyse van de genetische diversiteit met de ERIC-PCR toonde een klonale verwantschap aan tussen pathogene *E. coli* stammen die dezelfde virulentiefactoren dragen en hetzelfde antibioticumresistentieprofiel hebben. De implementatie van deze epidemiologische onderzoeksgegevens in heel Cuba kan bijdragen aan het epidemiologische surveillance, preventie en controle van varken colibacillose.

In acht genomen dat de aanwezigheid van F4- en F18-specifieke antistoffen bij Cubaanse varkens een weerspiegeling is van de aanwezigheid en verspreiding van $F4^+$ ETEC of $F18^+$ ETEC/VTEC, werd in **Hoofdstuk 4** het voorkomen van F4- en F18-specifieke antistoffen in sera van 1044 gelten onderzocht. Data analyse gebeurde aan de hand van een 'random effect model' en een 'mixture model' in R (package "mixAK"; Komárek, 2009). Lage, gemiddelde en hoge aantallen F4-specifieke antistoffen werden gevonden in respectievelijk 67.6%, 26.8% en 5.6% van de gelten, terwijl lage en hoge aantallen F18-specifieke antistoffen respectievelijk werden teruggevonden in 66.4% en 33.6% van de onderzochte gelten. Dit toont aan dat $F4^+$ *E. coli* en $F18^+$ *E. coli* een zeer veel voorkomende enteropathogeen is in Cubaanse varkensbedrijven.

In **Hoofdstuk 5** werd onderzocht wat het voorkomen is van de genotypes en de allelen die bepalen of biggen gevoelig of resistent zijn voor F18$^+$ *E. coli* infecties. Om de gevoeligheid voor F4$^+$ *E. coli* infecties te bepalen, werd het *Xba*I polymorfisme in het *mucine 4* gen van het varken onderzocht bij varkens gekweekt in de Villa Clara provincie in Cuba. Het *Xba*I resistente genotype werd het meest teruggevonden (0.66), terwijl de heterozygoot of homozygoot gevoelige genotypes minder frequent voorkwamen (respectievelijk 0.31 en 0.03). Gezien we een hoge seroprevalentie van F4-specifieke antistoffen in sera van jonge zeugen gevonden hebben in Cuba, kunnen we besluiten dat het *Xba*I polymorfisme in het *mucine 4* gen niet kan gebruikt worden als betrouwbare merker om genetische resistentie tegen F4$^+$ *E. coli* op te sporen.

Om de gevoeligheid voor F18$^+$ *E. coli* infecties te achterhalen, werd het *Cfo*I polymorfisme in het *FUT1* gen onderzocht. Varkens met het F18-resistente genotype werden niet frequent teruggevonden (0.13), terwijl de heterozygoot of homozygoot gevoelige genotypes het meest frequent voorkwamen (respectievelijk 0.27 en 0.60). Het hoge aantal F18$^+$ *E. coli* gevoelige biggen correleert met de hoge prevalentie van F18$^+$ *E. coli* en F18-specifieke antistoffen in de Cubaanse varkenspopulatie. Genetische selectie van biggen met het F18$^+$ *E. coli* resistente genotype zou dus in de toekomst een maatregel kunnen zijn om diarree en slingerziekte bij varkens in Cuba terug te dringen. Deze strategie wordt nog niet aanbevolen voor F4$^+$ *E. coli* gezien het *Xba*I polymorfisme in het *mucine 4* gen geen betrouwbare merker is om resistentie tegen F4$^+$ *E. coli* terug te vinden.

Tot slot worden de algemene discussie en de toekomstperspectieven weergegeven in **Hoofdstuk 6**. Er wordt voorgesteld dat een hogere productie-efficiëntie van het Cubaans Bedrijf voor varkensproductie kan bereikt worden door de verbetering van bewakings-, preventie- en controleprogramma's van infectieuze diarree, en voornamelijk van collibacillose bij jonge biggen. De Cubaanse overheid zou het belang moeten erkennen van de identificatie van de enteropathogenen en van het opsporen van antibioticumresistenties door de diagnostische laboratoria. Op korte termijn zou vaccinatie tegen *E. coli*, het invoeren van efficiënte antibiotica en de verbetering van management maatregelen zeer gunstig kunnen zijn om neonatale en speendiarree bij jonge biggen in Cuba in te perken. Op lange termijn zou selectie voor F4- en F18-resistente biggen en investeringen in de faciliteiten van de Cubaanse varkensbedrijven sterke voordelen kunnen opleveren om F4$^+$ en F18$^+$ *E. coli* infecties te bestrijden.

Resumen

La diarrea pre- y post-destete de los cerdos son enfermedades multifactoriales que afectan negativamente la eficiencia de la producción en granjas porcinas a nivel mundial. A menudo los veterinarios del sector porcino de Cuba se quejan de la escasa información sobre la epidemiología de la diarrea y las limitaciones para la identificación específica de enteropatógenos. Por lo tanto, se llevan a cabo medidas de control insuficientes que resultan en un aumento de la incidencia de la diarrea porcina.

La presente tesis realiza una actualización del conocimiento epidemiológico sobre la etiología infecciosa de la diarrea en cerdos jóvenes de Cuba a través de la identificación diferencial de enteropatógenos en cerdos recién nacidos, lactantes, y recién destetados. Este estudio epidemiológico identificó a *Escherichia coli* como un enteropatógeno común por lo que la tesis se enfoca más en las infecciones por *E. coli*. En primer lugar se determinó la resistencia a los antibióticos y la relación genética de cepas cubanas de *E. coli* patógenas. Posteriormente se evaluó la seroprevalencia de anticuerpos contra las fimbrias F4 y F18 de *E. coli*. Por último se analizó la susceptibilidad genética de los cerdos de Cuba para la colonización entérica por *E. coli* $F4^+$ y $F18^+$.

El **primer Capítulo** es una revisión bibliográfica dedicada a la producción porcina en el contexto cubano y a la epidemiología de los principales enteropatógenos causantes de diarrea en cerdos jóvenes en todo el mundo. También se discutió sobre la identificación diferencial de enteropatógenos y se introdujo el término epidemiológico ¨tipos mixtos¨ que se refiere a la etiología infecciosa combinada que la diarrea porcina puede presentar.

El **Capítulo 2** se llevó a cabo para obtener información sobre la etiología infecciosa de la diarrea porcina pre- y post-destete en la provincia de Villa Clara, Cuba. Fueron analizados contenidos intestinales de cerdos diarreicos por medio de cultivo, microscopía, y técnicas inmunológicas o basadas en el ADN. Por lo menos un enteropatógeno se detectó en el 64,4% y en el 42,2% de los lechones lactantes y destetados, respectivamente. *E. coli* enterotoxigénica (ETEC) fue significativamente el patógeno más frecuente, y la mayoría de los virotipos fueron STa^+/STb^+ o $F4^+/STa^+/STb^+$. La ocurrencia global del resto de los patógenos fue del 10% para el virus de la gastroenteritis transmisible y *Cryptosporidium parvum*, 6,7% para rotavirus A e *Isospora suis*, 5,6% para *Clostridium perfringens* α-toxigénico, 3,3% para *E. coli* verotoxigénica (VTEC), y 2,2% para *Salmonella enterica* subespecie *enterica* serovar Newport. El virus de la diarrea epidémica porcina, *C. perfringens* β-toxigénico, *Eimeria* spp., y helmintos no fueron identificados. Doce de los 48 lechones

positivos a enteropatógenos (25%) estaban infectados con más de un patógeno y ETEC estuvo presente en 10 de las 12 infecciones mixtas. Estos resultados demuestran que varios enteropatógenos, solos o como parte de una infección mixta, están asociados con la diarrea porcina pre- y post-destete en la provincia de Villa Clara, Cuba.

Dado que *E. coli* fue el enteropatógeno más frecuente de los cerdos diarreicos en Cuba, este patógeno se estudió más detalladamente en los capítulos siguientes.

En el **Capítulo 3** se determinó el perfil de resistencia a los antibióticos y la relación genética de *E. coli* patógenas aisladas de lechones que sufrían diarrea. Las mayores tasas de resistencia se mostraron ante antibióticos tradicionalmente administrados en granjas porcinas cubanas: tetraciclina (69%), ampicilina (54%), compuestos de la sulfonamida (50%), y kanamicina (50%); además el 65% de los aislados fueron resistentes a múltiples antibióticos. El ERIC-PCR reveló un alto grado de polimorfismo en las secuencias de ADN de *E. coli* así como relación entre los aislados $F4^+/STa^+/STb^+$ o $F18^+/LT^+/STb^+$ detectados en granjas porcinas diferentes, y entre aislados STb^+, STa^+/STb^+, $F4^+/STa^+/STb^+$ o $F18^+/Stx2e^+$ procedentes de una misma cochiquera. *E. coli* patogénicas, genéticamente diversas o genéticamente relacionadas, así como altamente susceptibles al ácido nalidíxico, ciprofloxacina, gentamicina, amikacina, cloranfenicol, cefalosporinas, amoxicilina-ácido clavulánico y trimetoprima están asociadas con la diarrea de los lechones en la provincia de Villa Clara, Cuba. El análisis de la diversidad genética por medio de ERIC-PCR demostró relación clonal entre *E. coli* patógenas que portaban los mismos factores de virulencia y similar perfil de resistencia a los antibióticos. La implementación de los resultados de esta investigación epidemiológica en toda Cuba puede contribuir a la vigilancia, prevención y control de la colibacilosis porcina.

Teniendo en cuenta que los anticuerpos séricos específicos para F4 o F18 en los cerdos cubanos podrían reflejar la presencia y propagación de ETEC $F4^+$ o ETEC/VTEC $F18^+$, en el **Capítulo 4** se determinó la prevalencia de anticuerpos específicos contra F4 y F18 en el suero de 1044 cerdas jóvenes. Para el análisis de datos se emplearon modelos de efectos aleatorios así como modelos de mezcla en R (paquete "mixAK"; Komárek, 2009). Niveles bajos, moderados y altos de anticuerpos específicos contra F4 se encontraron en el 67,6%, 26,8% y 5,6% de las cerdas, mientras que el 66,4% y 33,6% presentaron niveles bajos y altos de anticuerpos específicos contra F18, respectivamente. En este estudio se ha mostrado que *E. coli* $F4^+$ y $F18^+$ son muy frecuentes como enteropatógenos potenciales en cochiqueras cubanas.

El **Capítulo 5** se llevó a cabo para conocer en primer lugar la frecuencia de los genotipos y los alelos que determinan la susceptibilidad o resistencia a las infecciones por *E coli* F18^{+}. Para investigar la susceptibilidad a infecciones por *E. coli* F4^{+} se determinó el polimorfismo *Xba*I del gen porcino *mucin 4* en cerdos criados en la provincia Villa Clara, Cuba. El genotipo resistente a la digestión por *Xba*I fue detectado frecuentemente (0,66), y los genotipos susceptibles heterocigoto u homocigoto fueron menos frecuentes (0,31 y 0,03, respectivamente). Dado que hemos encontrado que hay una alta seroprevalencia de anticuerpos específicos contra F4 en suero de cerdas jóvenes en Cuba, llegamos a la conclusión de que el polimorfismo *Xba*I en el gen *mucin 4* no puede ser utilizado como un marcador fiable para determinar la resistencia genética a infecciones por *E. coli* F4^{+}. Los cerdos portadores del genotipo resistente a F18 no fueron encontrados con frecuencia (0,13), mientras que los genotipos susceptibles homocigoto o heterocigoto ocurrieron con mayor frecuencia (0,27 y 0,60, respectivamente), coincidiendo con informes anteriores de una alta prevalencia de *E. coli* F18^{+} y de anticuerpos específicos contra F18 en el rebaño porcino de Cuba. Por lo menos para el control de *E. coli* F18^{+} se podría considerar un mejoramiento genético de los cerdos teniendo en cuenta el genotipo resistente como una manera de controlar la diarrea porcina o la enfermedad edemática en Cuba. Esta estrategia no se recomienda para F4 debido a que el polimorfismo *Xba*I en el gen *mucin 4* no parece ser un buen marcador de resistencia a infecciones por *E. coli* F4^{+}.

Por último, la discusión general y las perspectivas futuras se presentan en el **Capítulo 6**. Una mejor eficiencia productiva de la Compañía Cubana de producción de carne de cerdo podría lograrse mediante la mejora de los programas de vigilancia, prevención y control de la diarrea infecciosa en los cerdos jóvenes, en particular colibacilosis. Las autoridades veterinarias cubanas deberían incentivar la identificación de enteropatógenos y la vigilancia de resistencia a los antibióticos por los laboratorios de diagnóstico veterinario. En el corto plazo, la vacunación contra *E. coli*, la introducción de antibióticos eficaces y la mejora de las prácticas de gestión parecen ser ventajosos para controlar la diarrea porcina pre- y post-destete en Cuba. En el largo plazo, la cría de cerdos resistentes a F18 o F4, así como inversiones en instalaciones, será necesario.

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Curriculum vitae

Pedro Yoelvys de la Fé Rodríguez was born on the 21st of March 1978 in Santa Clara, Villa Clara, Cuba. He coursed the high school at the Pre-University Institute of Exact Sciences from 1992 to 1995 in Santa Clara. Pedro graduated Doctor in Veterinary Medicine (DVM, awarded with Gold diploma) at Universidad Central ¨Marta Abreu¨ de Las Villas (UCLV), Cuba on July 2001, and finished the Master in Parasitology at Institute of Tropical Medicine ¨Pedro Kourí¨, Havana city, Cuba on March 2004.

Professional experience

From September 2001, he has been teaching at the Faculty of Agriculture and Animal Sciences, UCLV, Cuba. Teaching assistant of the undergraduate courses Veterinary Practice, Biological Agents (Parasitology-Microbiology-Immunology), Veterinary Epidemiology, and Zoonotic Diseases as member of the academic staff. Postgraduate lecturer in the master program Advanced Animal Health and in the specialized course of Veterinary Clinics. He has supported the Cuban company for pork production and the provincial Veterinary Diagnostic Laboratory on aspects related with animal health. Head of the discipline Preventive Veterinary Medicine at UCLV.

In April 2007, he began a joint (sandwich) PhD in the field of "Swine diarrheic diseases" at the Department of Veterinary Medicine and Zootechnics, Faculty of Agriculture and Animal Sciences, UCLV, Cuba and the Laboratory of Immunology, Department of Virology, Parasitology, and Immunology, Faculty of Veterinary Medicine, Ghent University, Belgium.

Publications in peer-reviewed journals

Publications related with the PhD project in top 50% journals of Veterinary Sciences

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